UNIVERSITY OF ALBERTA

EFFECTS OF PREGNANCY, ESTRADIOL AND PROGESTERONE ON PLASMA ATRIAL NATRIURETIC PEPTIDE (ANP) AND THE PRESSOR RESPONSIVENESS TO ANGIOTENSIN II (AII) IN THE RAT

BY

KELLI ANN NOVAK

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ABSTRACT[1]

The responses to infused angiotensin II (AII) were measured in conscious, pregnant, Long-Evans rats. A significant reduction in the pressor response was found at days 7, 14 and 21 of pregnancy. The reflex decrease in heart rate was observed at days 7 and 14, but was lost by day 21 of pregnancy.

Virgin, ovariectomized rats were injected s.c. for 10 days with either estradiol, progesterone or a combination of both. None of the hormone-injected groups showed any significant deviation in their pressor or heart rate responses to AII when compared with the saline-injected controls.

Plasma ANF levels increased significantly at day 7, remained slightly elevated at day 14, but fell to pre-pregnant levels at day 21 of pregnancy. ANF levels were also elevated in the estradiol-injected rats, but were normal in the progesterone-injected and estradiol plus progesterone-injected animals.

It is concluded that a) the rat is a valid model of human pregnancy in which to study blood pressure regulation, b) the baroreceptor reflexes may be impaired during pregnancy, c) neither estradiol nor progesterone is directly responsible for the reduced pressor response to AII, d) ANF levels depend on the balance of estradiol and progesterone and e) ANF may contribute to the reduced pressor response to AII in early pregnancy, but is unlikely to play a role near term.

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CHAPTER 1

INTRODUCTION

The study of cardiovascular and endocrine changes associated with pregnancy is surrounded by many inconsistencies, paradoxes and unknowns. Very little is understood of the cardiovascular and endocrine changes associated with normal pregnancy. This makes it very difficult to determine the causes of abnormalities in these areas during pregnancy and to treat them.

It is known that during normal human and rat pregnancy, blood pressure falls despite progressive increases in extracellular fluid (ECF) volume and blood volume (1); in the non-pregnant state, these increases in ECF and blood volumes would certainly bring about a rise in blood pressure. There is also increased sodium appetite (1) and increased renal sodium retention during both normal human and normal rat pregnancy (1,2); despite this, plasma osmolality is reduced (3). If, during pregnancy, blood pressure does rise, a pathological condition known as pregnancy-induced hypertension (PIH) ensues. Curiously, this rise in blood pressure is accompanied by a paradoxical decrease in blood volume (4).

During normal pregnancy there is a refractoriness to the pressor response to injected angiotensin II (AII) (5,6,7,8). This reduction in the pressor response to AII is noted as early as the first trimester of human pregnancy (9,10). In PIH the refractoriness to injected AII is lost, even before the onset of frank hypertension (9).

If this state of PIH is further complicated by proteinuria, a more serious

pathological condition is brought about, known as pre-eclampsia (PE). Both of these pathological states are very dangerous to both mother and fetus, and can have disastrous consequences if left untreated. In fact, PIH ranks among the top three causes of maternal death. Virtually nothing is known about the underlying cause of PIH which is not surprising considering how little is known about blood pressure regulation in normal pregnancy.

In order to determine the mechanisms behind these important adaptations to pregnancy, attention must be placed on the reflex hormonal responses to changes in blood pressure and volume. The renin-angiotensin-aldosterone system (RAAS) is of particular interest for two reasons: First, because of its intimate involvement in blood pressure and volume homeostasis, and second, because of the changes that occur in pressor responsiveness to AII in both normal and hypertensive pregnancies.

Much attention has been paid to the RAAS during pregnancy, but not all of the answers have been found. It is now well established that the pressor response to injected AII is reduced as early as the tenth week of gestation, and persists to term in a normal human pregnancy, and that this refractoriness to AII is lost in pregnancy complicated by hypertension (PIH). The primary mechanism of these changes in pressor response is not known. It is clear, that in order to understand and treat PIH, we must gain a better understanding of what occurs during a normal pregnancy.

To do this, a suitable animal model must be found. At present, there is no universally accepted animal model for the study of cardiovascular and/or endocrine parameters during pregnancy. Pregnancy-induced refractoriness to AII and other vasoactive substances has been studied in several animal species

(6,7,8,11,12,13). Unfortunately, the results have been inconsistent, contradictory, and masked by the use of anesthetics and restraints. Furthermore, in no case has the refractoriness to AII been demonstrated during the first trimester of pregnancy, a characteristic which I feel is important to establish in a potential model of human pregnancy.

This brought about the first aim of the present study: to establish an animal model for the study of vascular refractoriness to AII during pregnancy. We chose the rat as a possible candidate. Rats are reasonably inexpensive to obtain and house, and are hardy animals, able to withstand the stresses of surgery and other manipulation with a minimum of difficulty. Previous work in this field had been attempted on the rat. However, the results were conflicting. Paller (7) found in rats that the pressor response to AII was unchanged at day 10 of pregnancy, but was reduced by day 15. Similarly, Conrad and Colpoys (14) found a significant reduction at day 20 of rat pregnancy, but no change at either day 12 or 16. We then set out to determine whether the pressor response to injected AII would be significantly reduced within the first trimester of rat pregnancy, using only conscious, unrestrained animals, remote from the effects of anesthesia or surgical trauma.

Attention was then focussed toward determining the underlying mechanism of the reduced pressor responsiveness to AII. Two candidates which immediately come to mind are progesterone and estradiol. Both of these hormones increase several-fold during pregnancy, and are known to interact with the RAAS (15). Progesterone, on one hand, has been shown to cause fluid retention and expansion of the extracellular fluid space (1), while on the other hand, has also been shown to be natriuretic and to inhibit sodium retaining effects of

mineralocorticoids (16). Estrogen has been shown to induce sodium retention (1).

The situation with respect to these hormones and blood pressure control is unclear. In humans, the effects of progesterone are not established with certainty (9,17). In rats, some investigators have shown progesterone to cause a decrease in the pressor response to AII (18,19,20), while others have found no such decrease due to progesterone (17,21). Similarly, there are reports of estrogen causing a reduction in the pressor response to AII (13,21,22,23,24), while others report that estrogen treatments did not significantly alter the pressor response to AII (17,18,20,25). The vast discrepancies in the results obtained by these investigators undoubtedly lie in the differences of the methods employed. Of particular consequence is the use of anesthesia and surgery without adequate recovery time before performing the experiments. It is no secret that anesthetics alter cardiovascular parameters for a significant amount of time following administration (26).

This brought about the second aim of the present study: to determine whether or not progesterone and estradiol are involved in the altered vascular responsiveness to AII associated with pregnancy. Only conscious, unrestrained rats, remote from the effects of anesthesia and surgical trauma would be used for this.

No study of vascular responsiveness to AII would be complete without looking at the change in heart rate which occurs concurrent to the change in blood pressure. In the non-pregnant state, AII causes an increase in blood pressure which triggers the baroreceptor reflex resulting in bradycardia in order to restore blood pressure to its initial value. While much attention has

been paid to the pressor response to AII during pregnancy, very little information is available regarding the concurrent heart rate response to AII during pregnancy.

This provided the third aim of the present study: to determine whether the heart rate response to AII is at all affected by pregnancy, estradiol or progesterone.

Another obvious candidate in the search for the mechanism of pregnancy-induced changes in blood pressure control and fluid homeostasis, is atrial natriuretic peptide (ANP). ANP is a known antagonist of AII and has been shown to reduce the pressor response to AII in nonpregnant rats and humans (27). Much of the evidence reported, to date, indicates that plasma ANP levels are increased in human pregnancy. There is, however, a problem; ANP is natriuretic, not salt retaining. Furthermore, it is unclear in the literature whether or not ANP levels increase during normal rat pregnancy. Few reports exist in the area of ANP levels during normal rat pregnancy and those that do, contradict one another, and are masked by major differences in blood collection source and technique, and assay type (28,29,30,31).

This led to the fourth aim of this study: to determine whether plasma ANP levels rise or fall during normal rat pregnancy. If elevated, one could infer that ANP may be responsible for the reduced pressor responsiveness to AII associated with pregnancy.

An interesting proposal, made by Back, Forssmann and Stumpf (32), led to the final aim of this study. They found that atrial myocytes specifically accumulate estradiol, and proposed that steroid hormones play a role in the regulation of ANP synthesis and/or release. If this is indeed the case, one

would wonder about the effects of altered progesterone and estradiol levels, such as in pregnancy, on the plasma levels of ANP.

The fifth, and final aim of this study, therefore, was to determine whether estradiol and progesterone had any effect on plasma levels of ANP.

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CHAPTER 2

EFFECTS OF PREGNANCY, ESTRADIOL AND PROGESTERONE ON THE PRESSOR RESPONSIVENESS TO ANGIOTENSIN II[2]

INTRODUCTION

Normal human pregnancy is characterized by profound changes in fluid and electrolyte balance and blood pressure homeostasis. Despite progressive volume expansion, sodium retention, and elevated circulating angiotensin II (AII) levels, blood pressure falls (18), and the pressor responsiveness to injected AII is reduced as early as the first trimester of pregnancy (9). The mechanism of this refractoriness to AII is unknown.

In contrast, pregnancy-induced hypertension (PIH) is characterized by a reduction in plasma volume (8), and hypersensitivity to the actions of AII (9).

Very little is known about the causes of PIH and this is not surprising since very little is understood about blood pressure regulation in normal pregnancy. It is important to gain a better understanding in these areas since PIH ranks among the top three causes of maternal death. In order to do this, attention must be focussed on establishing a valid animal model to study.

-2. A version of this chapter has been: submitted for publication (subject to minor revision). Novak & Kaufman 1991. American Journal of Physiology.

Studies in the area of pressor responsiveness to AII during pregnancy have been attempted on several different animal species (6,16,21,24,26,28). However, no universally acceptable animal model has, as yet, been determined, and, in no case has refractoriness to AII been demonstrated during the first trimester in these animals. If the rat can be established as a valid model for human pregnancy, there are several advantages which make it superior to other choices; it is a hardy animal, inexpensive to obtain and house, and it has a relatively short gestation period (22 days), making pregnancy easy to study.

Many of the possible mechanisms of the decreased responsiveness to AII have been tested experimentally, and have been eliminated, these include; prior occupancy of the AII receptors (24), and down-regulation of the receptors (24). The question as to whether prostaglandins play a role remains unanswered as conflicting reports also exist in this area. Some investigators indicate that prostaglandins do play a role in the reduced pressor responsiveness to exogenous AII (7,24), while other investigators have found that the pressor response was not improved by inhibition of prostaglandin synthesis (4). The result of all of this, is that the primary determinant for the reduced vascular resistance to infused AII during pregnancy remains open to question.

There is similarly much controversy in the literature regarding the effects of estradiol and progesterone on the pressor response to AII during pregnancy. There are reports of estrogen treatments causing a reduction in the pressor response to AII (12,25,26,27,29) while other investigators report that estrogen treatments have no effect (3,11,22,23). Some reports indicate that progesterone treatments cause a reduction in the pressor response to AII (11,20,22) while other investigators report that progesterone does not alter

the pressor response to AII (3,26). It is not surprising that so much work has been focused in this area, since these hormones increase several fold during pregnancy, progesterone is known to exhibit vasorelaxing properties, and both progesterone and estrogen are known to interact with the renin-angiotensin-aldosterone system (RAAS) (10).

In order to establish possible mechanisms behind the reduction in pressor response to AII during pregnancy, this study was designed to do two things:

1) establish the rat as an appropriate model for human pregnancy by confirming that in conscious, unrestrained, pregnant rats, the pressor response to injected AII does indeed fall in the first trimester, and;

2) to study the effects of estradiol and progesterone on the pressor response to injected AII in conscious, unrestrained animals in an attempt to clear up the controversy that exists in this area.

METHODS[3]

Animals: Virgin female Long-Evans (LE) rats weighing between 200 and 225 grams were obtained from Charles River Canada, St. Foy, Quebec. They were held at least one week prior to surgery, in a temperature and humidity controlled room with 12 hours of light (0700 to 1900) and 12 hours of darkness (1900 to 0700). Surgery was not performed until the rats weighed between 240 and 280 grams. They were then caged individually following surgery. Animals for experiments A & B were maintained on 0.3% sodium chow (Bioserv Inc., New Jersey), those for experiment C were maintained on normal rat chow.

Surgery - Experiments A & B: All rats were surgically prepared with cannulae, under sodium pentobarbital anesthesia (41.6 mg/kg B.wt.). Each rat was also given a standard dose of 0.2 ml atropine sulphate (0.6 mg/ml), and 0.22 ml Derapen-C (200,000 IU penicillin-G/ml) pre-operatively. One silastic cannula (0.020 in. ID, 0.037 in. OD), and one polyethylene cannula (PE10), were non-occlusively implanted into the inferior vena cava (IVC), using the method described by Kaufman (14). One polyethylene cannula (PE10) was similarly implanted non-occlusively into the abdominal aorta, approximately 1 cm caudal to the branching of the renal arteries. The method of aortic cannulation was similar to that described by Kaufman (14) with some modifications. In summary,

-3. A more detailed description of the Methods section for this chapter is provided in Appendix A.

a PE10 cannula, previously prepared with a U-shaped "crook" and a cuff near the insertion end, was inserted into the aorta at a point midway between the branching of the renal arteries and the bifurcation of the aorta. The cannula was advanced until the base of the "U" lay at the puncture site. A 6-0 prolene ligature was used to lash the base of the "U" to the tunica adventitia, and two 4-0 silk ligatures were used to lash the cannula to the psoas muscle, with one suture above and one below the cuff.

The cannulae were exteriorized at the nape of the neck via 1.5 cm lengths of stainless steel tubing, filled with approximately 200 ul of heparinized saline (10,000 units/litre) to maintain patency, and sealed with silastic caps. The aortic cannula also contained approximately 20 ul of polyvinylpyrrolidone solution (8 mg PVP/10 ml heparinized saline) to prevent the arterial blood from moving up the cannula and coagulating. The male rats did not disturb the metal access tubing during mating. These cannulae were used to record blood pressure and heart rate, and for blood sampling and the infusion of angiotensin II (AII). The age control animals for experiment A, and the animals to be used in experiment B, also underwent bilateral ovariectomy at this time.

Post-operatively, all animals were checked daily to ensure that they were eating and drinking adequately, and that the incisions were healing well. Body weights were taken 7 days post-operatively to ensure that the initial weight had been regained. Animals were not used for experimentation until pre-operative weight was achieved. Any animals noted to be in distress, or which did not gain weight at an appropriate rate (2 gm/day) were not used for experimentation.

Surgery - Experiment C: The animals were prepared by the method described by Nakamura et al (22), with some modifications. In summary, all animals underwent bilateral ovariectomy under sodium pentobarbital anesthesia (41.6 mg/kg B.wt.) administered intraperitoneally. They were allowed to recover for 10 days while receiving hormone injections (one per day).

Following the tenth injection, the animals were anesthetized with chloroform, and cannulae were placed in the femoral artery (PE10) and vein (PE50). After surgery the animals were placed in restraining cages and allowed to recover for a minimum of 60 minutes before AII testing was performed.

Blood Sample Collection/Analysis: A short (6 cm) extension of PE50 tubing was connected to the larger IVC cannula at the nape of the neck. Blood samples (1 ml) were collected into dry, tuberculin syringes and then immediately transferred into polypropylene microcentrifuge tubes containing 2 ul of Trasylol plus 2 ul of 13.8% w/v EDTA. Blood was centrifuged at 4°C for 10 min at 1,000 g and the plasma was then stored in polypropylene tubes at -43°C until analysis. The plasma levels of estrogen and progesterone were measured by radioimmunoassay (RIA) using kits from Amersham Life Science Products.

Measurement of Blood Pressure and Heart Rate Responses to AII:

For experiments A and B, the animals were handled briefly in order to hook up cannulae extensions to the stainless steel tubing at the nape of the neck. The animals were then returned to the metabolic cages where they could move freely. Cannulae were then flushed open with approximately 300 ul of heparinized saline (10,000 units/litre). The arterial cannula was then

connected to a Statham pressure transducer (Gould Inc.) for the continuous recording of heart rate and mean arterial pressure. The PE10 IVC cannula was connected to an infusion pump, with normal saline infusing at a rate of 2 ml/hr. This rate of infusion was noted, by the current authors, to cause no significant perturbation in BP, HR or CVP. The rats were allowed 20-40 minutes to settle before AII infusions were commenced. The saline infusion was then briefly interrupted, and 100 ng of AII in 10 ul normal saline was infused via the PE10 IVC cannula at the same rate (2 ml/hr), after which the normal saline infusion was immediately resumed. The pressor response was observed and the animals were allowed to return to resting BP prior to the administration of the next bolus (10-30 minutes). A second infusion of AII (100 ng in 10 ul) was then administered in the same manner. Preliminary results using a dose response curve showed that a minimum dose of 100 ng AII was necessary in order to consistently give a pressor response throughout all stages of pregnancy. Doses of 10 ng, 20 ng and 50 ng did not evoke significant pressor responses in the latter stages of pregnancy.

For the animals in experiment A, the AII dosage was then corrected for body weight (400 ng AII /kg B.wt.) and two infusions were given in the same manner.

Upon return to resting BP following the final dose of AII, the rats were disconnected from the transducers, the cannulae were filled with heparinized saline and capped closed to maintain patency.

For experiment C, following the 60 minute recovery period the femoral arterial cannula was connected, via extension tubing, to a Statham pressure transducer for the continuous recording of heart rate and mean arterial pressure. The venous cannula was connected to an infusion pump, with normal

saline infusing at a rate of 2 ml/hr. AII (100 ng in 10 ul) was then administered via the infusion pump as described for experiments A & B above.

Following the final dose of AII, the rats were sacrificed with an overdose of sodium pentobarbital.

Experimental Protocol

A) Blood Pressure/Heart Rate Responses in Pregnancy:

Following surgery, the rats were allowed a full 7 days recovery. They were then transferred into metabolic cages and allowed 24 hours to acclimatize. Resting blood pressure and pressor responses to AII were recorded in the manner described above. Vaginal smears were obtained to determine the stage of the estrous cycle. Vaginal smears were repeated every morning until proestrous was determined, at which time the female was placed with one male LE rat for a period of 36-48 hours. Day 1 of pregnancy was considered to be the first full day following initial placement together. The procedure for measuring pressor responses to AII and resting BP was then repeated, on the same rats, on days 7, 14 and 21 of pregnancy. Confirmation of pregnancy was made with successful parturition. For those animals whose cannulae failed prior to parturition, pregnancy was confirmed by post-mortem examination. The attrition rate of the cannulae increased greatly in the later stages of pregnancy.

A group of bilaterally ovariectomized females served as age controls and were tested when their post-operative ages were equivalent to pre-pregnancy, and days 7, 14 and 21 of pregnancy.

B) Blood Pressure/Heart Rate Responses in Hormone Treated, Ovariectomized Rats:

The rats were allowed 48 hours recovery from surgery. A 1.0 ml blood sample was then taken prior to commencement of hormone injections. Hormone dosage regimens were based on those used by Nakamura et al (22). Injections were administered subcutaneously into the loose skin of the back. The steroids were dissolved in sunflower oil. Eight rats were injected for 10 days with estradiol valerate (25 ug in 100 ul). Ten rats were given progesterone (gesterol 50 ug in 100 ul) for 10 days. Eight rats were given progesterone (gesterol 500 ug in 100 ul). Twelve rats were given a combination of 25 ug estradiol plus 50 ug progesterone in 200 ul, and eight rats were given a combination of 25 ug estradiol plus 500 ug progesterone in 200 ul. Eleven rats given subcutaneous injections of normal saline (100 ul per day) served as controls. The failure rate of the aortic cannulae was higher than that of the large IVC cannulae, hence more success was achieved in obtaining blood samples than in pressor response testing. Blood samples were taken even if pressor response testing could not be done due to aortic cannula failure.

The rats were then tested for resting BP and pressor responses to infused AII (100 ng in 10 ul normal saline) on day 12 post-operative (following the 10th injection), in the same manner as described above. A second blood sample was collected from each hormone-injected rat on the day following AII testing (following the 11th injection). The pressor response values for the hormone-treated animals were then compared with the values of the saline-injected controls.

C) Blood Pressure and Heart Rate Responses in Hormone Treated, Ovariectomized Rats - Chloroform:

The rats were allowed 24 hours recovery from surgery before commencement of hormone injections. Six rats were injected for 10 days with progesterone (gesterol 500ug in 100ul). Five rats were given 25ug estradiol plus 500ug progesterone in 200ul. Six rats, given subcutaneous injections of normal saline (100ul per day), served as controls. Following femoral artery and vein cannulation under chloroform anesthesia, the rats were tested for pressor responses to infused AII (100ng in 10ul).

Data Analysis: For all groups, the maximum elevation in blood pressure and accompanying decrease in heart rate produced by each of the two doses of AII was measured, and the mean response to a given dose was calculated for each animal. The rats were then grouped according to stage of pregnancy, age, or type of treatment. The mean resting BP, mean resting HR, and the mean pressor and HR responses to a given dose of AII, and the standard errors of the mean were calculated for each group. Analysis of Variance (ANOVA) between subjects, followed by the Newman-Keuls Test for multiple comparison was used to determine the significance of the changes in resting BP, resting HR, pressor responses, and heart rate responses to AII between the groups. The Unpaired Student's t-test was used to compare the chloroform treatments to conscious animals.

RESULTS[4]

Experiment A: Effect of Pregnancy:

There was no significant difference between the resting blood pressure prior to pregnancy (Pre) and at days 7 and 14 of pregnancy. However, blood pressure by day 21 of pregnancy was significantly lower than the pre-pregnant value ($p<0.01$) (Figure 1). The resting blood pressure of the ovariectomized age controls at the age equivalents of pre-pregnancy, and 7, 14 and 21 days of pregnancy showed no significant difference between the groups (Figure 1).

Figure 2 illustrates the pressor responses to both standard doses of AII (100ng per dose) and the AII dose corrected for body weight (400ng/kg B.wt), prior to pregnancy (pre) and during pregnancy, compared to the ovariectomized controls. A significant reduction in the pressor response was noted as early as day 7 of pregnancy for both the standard dose and the dose by body weight infusions of AII ($p<0.01$). Significant reductions in the pressor response were also noted at days 14 and 21 of pregnancy for both groups when compared to the pre-pregnant state (Figure 2). A further significant reduction was noted at 21 days of pregnancy when compared to both the 7 days pregnant state and the 14 days pregnant state ($p<0.01$). The ovariectomized age controls showed no significant change in pressor response over the course of the 21 days (Figure 2).

-4. Additional figures corresponding to the results contained in this chapter are provided in Appendix B.

Resting heart rate showed no significant deviation throughout the course of study in either the pregnant rats or the ovariectomized controls. However, the HR response during the infusion of AII was significantly lower ($p<0.01$) by day 21 of pregnancy (Figure 3). Though not significant, the change in HR did tend to follow the same pattern as the pressor response at days 7 and 14 of pregnancy (Figure 3). There was no significant change in the HR response of the ovariectomized controls over the course of the 21 days (Figure 3).

Experiment B: Effect of Hormone Treatments:

There were no significant differences in either blood pressure or pressor responses to infused AII (100 ng per rat) between any of the hormone treated groups and the saline-injected controls.

Resting heart rates were not significantly different in any of the hormone-treated groups. Nor was there any significant difference in the heart rate responses between the saline controls and any of the hormone-treated groups.

Experiment C: Effect of Hormone Treatments and Chloroform

Resting blood pressure was significantly lower ($p<0.05$) in the chloroform-anesthetized controls when compared to the conscious controls. Similarly, the pressor responses of the anesthetized rats was consistently lower than those of the conscious animals (Figure 4). However, hormone treatments did not affect the magnitude of these responses.

Resting heart rates were significantly lower in all chloroform-anesthetized groups when compared to their conscious counterparts, although there was no

difference between the treatment groups.

The heart rate responses to AII were lower in the anesthetized rats, when compared to their conscious counterparts (Figure 5). However, there was again no difference between the treatment groups.

Blood Sample Analysis: For the estradiol assay, the intra-assay coefficient of variation was 7%. The sensitivity of the assay was approximately 8 pg/tube, (32 pg/ml of serum), therefore values <32 pg/ml of serum were considered undetectable. The pre-treatment samples all showed undetectable levels of estradiol. The post-treatment samples for the estradiol-treated (25 ug/day) animals showed a mean of 147.4 ± 23.9 pg estradiol/ml (n=5). The progesterone-plus-estradiol-treated animals (500 ug/day plus 25 ug/day, respectively) the mean was 102.3 ± 18.8 pg estradiol/ml (n=7).

For the progesterone assay, the sensitivity of the assay was 0.08 ng/mlof serum. The pre-treatment samples for the progesterone group showed a mean of 6.24 ± 1.48 ng progesterone/ml (n=7), and for the progesterone plus estradiol group the mean was 16.92 ± 5.36 ng progesterone/ml (n=7). The post-treatment samples for the progesterone (500 ug/day) injected group showed a mean of 26.78 ± 7.05 ng/ml (n=6), and for the progesterone (500 ug/day) plus estradiol (25 ug/day) group the mean was 29.78 ± 8.46 ng/ml (n=6).

DISCUSSION

Most of the animal work in this field had been done in anesthetized, or recently anesthetized animals still recovering from the stress of surgery. There is a question as to what extent anesthesia itself as well as the fluid losses and replacement associated with surgery may influence the parameters under study. It is known that barbituate anesthetics alter systemic and renal hemodynamics, activate the renin-angiotensin system, impair cardiovascular function, and disturb renal blood flow and systemic and renal vascular resistance in rats (17). These factors have undoubtedly contributed to the conflicting results in the literature regarding the changes in cardiovascular response to AII that occur during pregnancy.

The first aim of this study, was therefore to confirm that the pressor response to injected AII does, indeed, fall in conscious, unrestrained pregnant rats within the first trimester of pregnancy. Paller (24) found, in recently anesthetized rats, that while there was no change in pressor activity at day 10, the response was blunted at day 15. Similarly, Conrad and Colpoys (4) found a significant reduction at day 20 in conscious, restrained rats, but no change at either day 12 or day 16. Ours is the first study to show a significant reduction in pressor response by as early as the seventh day, ie. first trimester, of rat pregnancy (Fig 2). This is an important finding because it better compares to the human condition. We also found a reduction in resting BP by the last week of pregnancy in the rat (Fig 1), which is in agreement with previous reports (1,28). This is also in agreement with the normal pregnant human condition in which a reduction in resting BP is seen

during pregnancy (18). Based on these findings, it is concluded that the rat is an appropriate model for the study of altered cardiovascular responsiveness to exogenous AII during human pregnancy.

Any agent, such as AII, which causes an increase in BP, will evoke the baroreceptor negative feedback reflex in an attempt to bring the BP back down. This is primarily achieved by a decrease in HR, which results in a decrease in cardiac output. The HR response during AII infusion was significantly lower by day 21 of pregnancy; in fact it was completely lost. This contradicts the findings of Massicotte et al (19) who found no change in heart rate response at 20 days gestation in their normotensive rats. This may be due either to our having used a different strain of rat, or to the different methods used to obtain their results; they used a relatively acute preparation of jugular vein and carotid artery cannulation under ether anesthesia, then allowed only 60-90 minutes recovery.

Of late, there has been much attention payed to the involvement of estrogens and progesterones in the mechanism of decreased pressor responsiveness to vasopressors during pregnancy. Controversy exists in the literature regarding the role of these sex steroids in the decreased pressor responsiveness to AII during pregnancy. We believe that this discrepancy exists because of the vastly different conditions under which the results were obtained. All three of the authors who reported that progesterone caused a decrease in the pressor response to AII obtained their results in anesthetized, or recently anesthetized and surgically traumatized animals (11,20,22), while Chesley & Tepper (3), who found no effect of progesterone on the pressor response to AII, obtained their results from conscious women. Similarly, all

of the reports of estrogen causing a reduction in the pressor response were obtained from conscious animals (12,25,27,29), while three of the four reports of estrogen having no effect on the pressor response were obtained from anesthetized animals (11,22,23). The fourth report indicating that estrogen had no effect of the pressor response to AII, was obtained from conscious women (3).

The second aim of the present study, was therefore to try to resolve the controversy over the possible effects of estrogen and progesterone on the pressor response to AII by carefully performing the experiments in conscious, unrestrained animals, remote from the effects of anesthesia and surgery.

In order to ensure stable (non-cyclic) levels of estrogen and progesterone, the animals were ovariectomized, then loaded with the hormones for a period of 10 days. The RIA indicated that, prior to hormone treatments, the estradiol levels were undetectable (<32 pg/ml). Following treatment, estradiol levels reached physiological levels slightly higher than those reported for proestrous and early pregnancy. Prior to treatments, progesterone levels were at the lower end of the physiological range (13). This is as expected since the blood samples were taken 48 hours post-ovariectomy, and it takes 96 hours for progesterone to clear the body (2). Progesterone levels after treatment (500 ug/day) were comparable to those reported for mid-metestrous, and late diestrous in an adult rat (13), and to those reported for day 21 of pregnancy (5), when the pressor response is most attenuated.

Our pressor response results are only partially in agreement with Nakamura et al (22). We did find that estradiol treatments did not significantly alter the pressor response to exogenous AII, as they did. This also agrees with the

findings of Chesley & Tepper (3) for conscious pregnant women. However, Nakamura et al (22) reported significant reductions in the pressor responses following progesterone (2000 ug/kg/day), and progesterone (2000 ug/kg/day) plus estradiol (100 ug/kg/day) treatments; our results do not indicate this.

In order to determine a possible cause for the discrepancy in our results compared with those of Nakamura et al (22), we attempted to reproduce their experimental procedure. Chloroform anesthesia was used and the femoral artery and vein were cannulated. We found the magnitudes of the pressor responses under these conditions to be significantly lower ($p<0.005$) than the pressor responses in our conscious, unrestrained animals (Figure 7). This indicates a significant upset to the cardiovascular system and vascular reactivity when still under the effects of anesthesia. It is, therefore, our belief that any experiments done in such close proximity to anesthesia and surgical trauma do not accurately represent the normal, conscious physiological situation. Furthermore, we still did not find that the progesterone-treated animals had a reduced pressor response to AII.

We have reproduced the experiments done by Nakamura et al (22) as closely as possible, and still have not been able to report a reduction in pressor response in the progesterone-treated animals. The reason for this difference may be species related (19), in that we used Long-Evans rats while Nakamura et al (22) used Wistar, or it may be due to the diet. We have found that the pressor response to AII is dependent on the quantity of sodium intake (unpublished results). Since sodium content is variable in normal rat chow, perhaps the group of rats used by Nakamura et al (22) was subject to a different sodium intake which could have altered cardiovascular and vascular

reactivity parameters.

Based on our results in both conscious animals and the chloroform anesthetized animals, it is concluded that neither estradiol nor progesterone has an effect on the pressor response to exogenous AII. These results are in agreement with those of Chesley and Tepper (3), who obtained their results from conscious women. Since our experiments were conducted in conscious, unrestrained animals remote from surgery and anesthesia, we believe our results provide a clearer indication of the normal physiological situation than any of the previous experiments where anesthetized, recently anesthetized, and/or restrained animals were used.

In summary, it is concluded that resting blood pressure is significantly reduced by the third week of pregnancy, and that the pressor response to exogenous AII is attenuated as early as the seventh day of pregnancy in the rat. Thus, the pregnant rat represents an appropriate model for the study of altered cardiovascular responsiveness to exogenous AII in the pregnant human. The reflex bradycardia response to exogenous AII is also significantly attenuated by day 21 of pregnancy. Furthermore, it is concluded that neither estradiol, progesterone, nor a combination of estradiol and progesterone is responsible for the attenuated pressor response to exogenous AII observed during pregnancy.

The primary determinant of the reduced pressor response to AII during pregnancy remains open to question. Very little attention has been given to the involvement of the baroreceptors in the cardiovascular response to exogenous vasoconstrictors during pregnancy. Some interesting aspects of the heart rate response were found in this study, and we feel that more careful consideration is needed on this integral component in the future.

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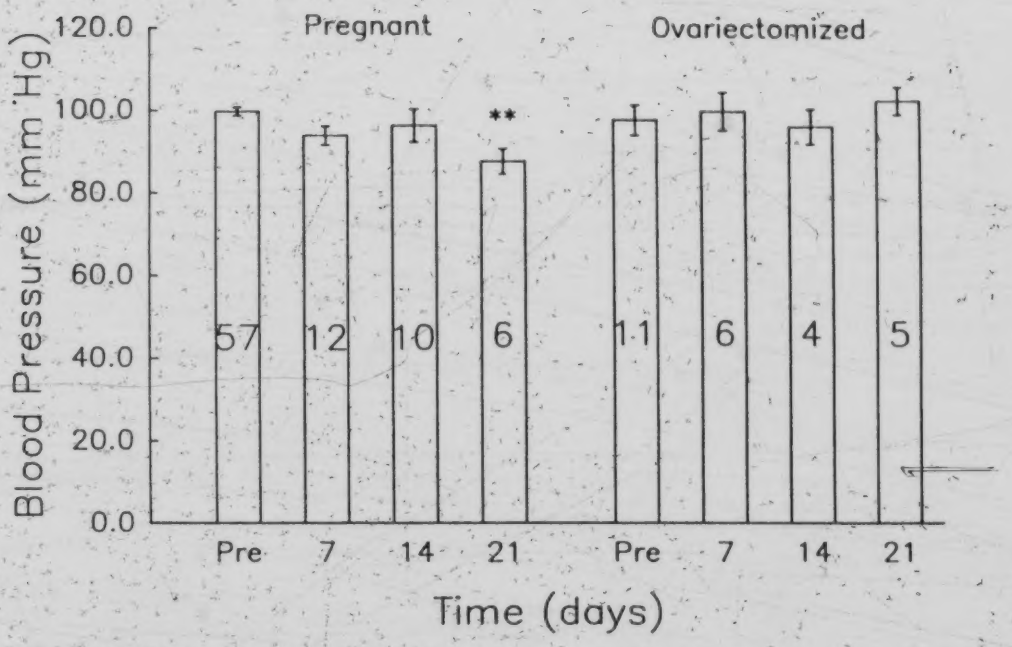

Figure 1 Resting arterial blood pressure of pregnant and acyclic female rats. Time refers to the stage of pregnancy. The ovariectomized rats were tested when their post-operative ages were equivalent to those of the pregnant rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes (**, $p<0.01$, when compared to Pre-pregnancy using Newman-Keuls Test).

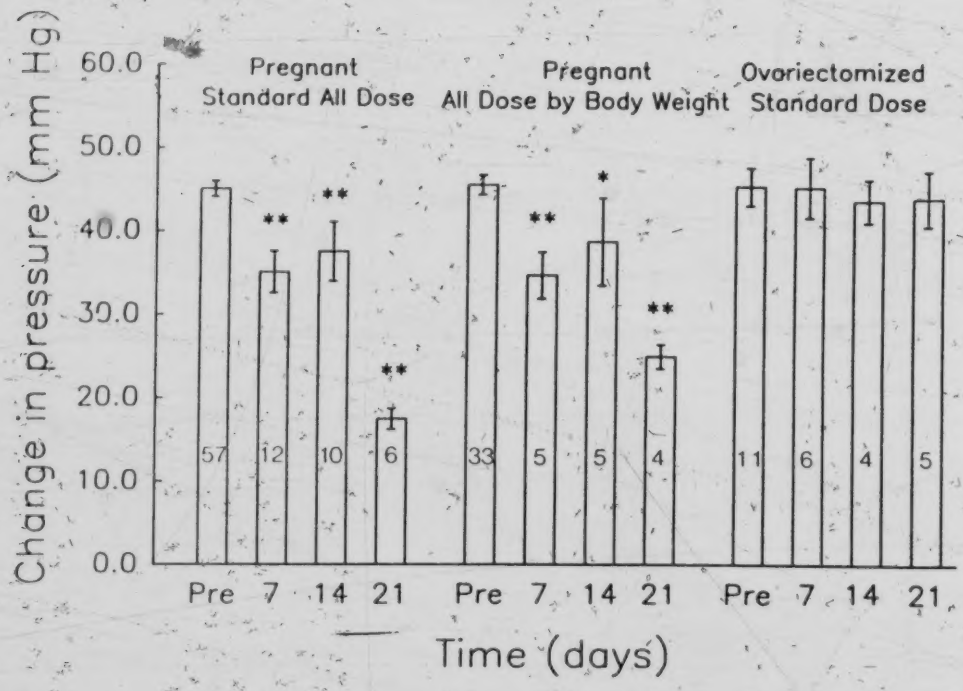

Figure 2 Comparison in increases in blood pressure for rats given a standard AII dose during pregnancy (100 ng in 10 ul), rats given the AII dose by body weight during pregnancy (400 ng/kg B.wt.), and ovariectomized controls receiving a standard AII dose (100 ng in 100 ul). Time refers to the stage of pregnancy or equivalent age. The acyclic rats were tested when their post-operative ages were equivalent to those of the pregnant rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes (*, $p<0.05$; **, $p<0.01$, when compared to Pre-pregnancy using Newman-Keuls Test).

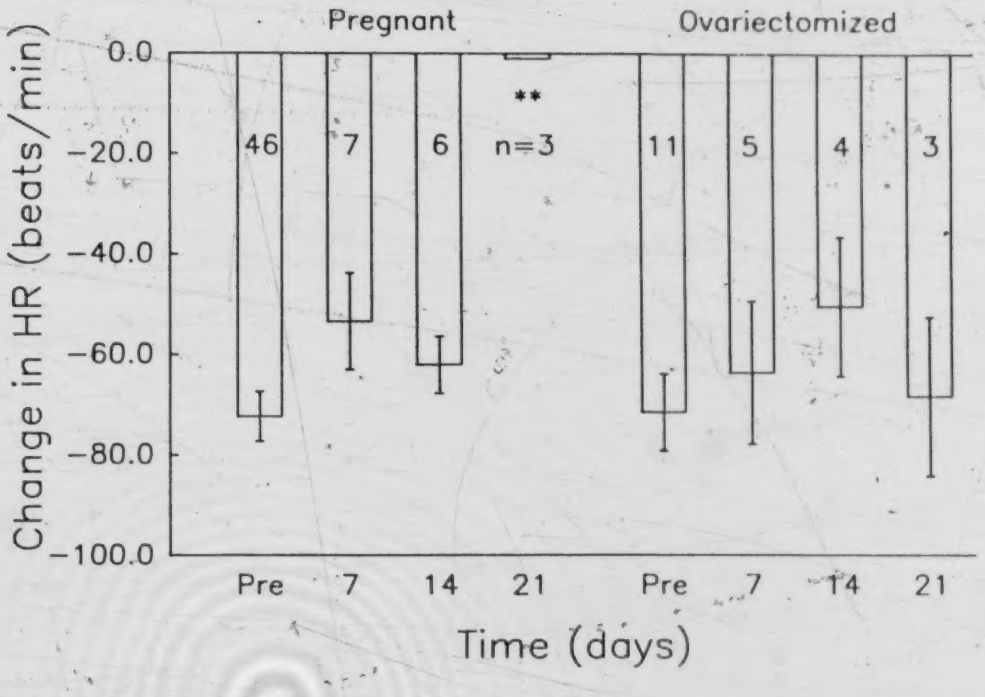

Figure 3 Comparison of changes in HR during infusion of standard AII dose (100 ng in 10 ul) between pregnant and acyclic animals. Time refers to the stage of pregnancy or equivalent age. The acyclic rats were tested when their post-operative ages were equivalent to those of the pregnant rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes. (**, $p<0.01$, when compared to Pre-pregnancy using the Newman-Keuls test).

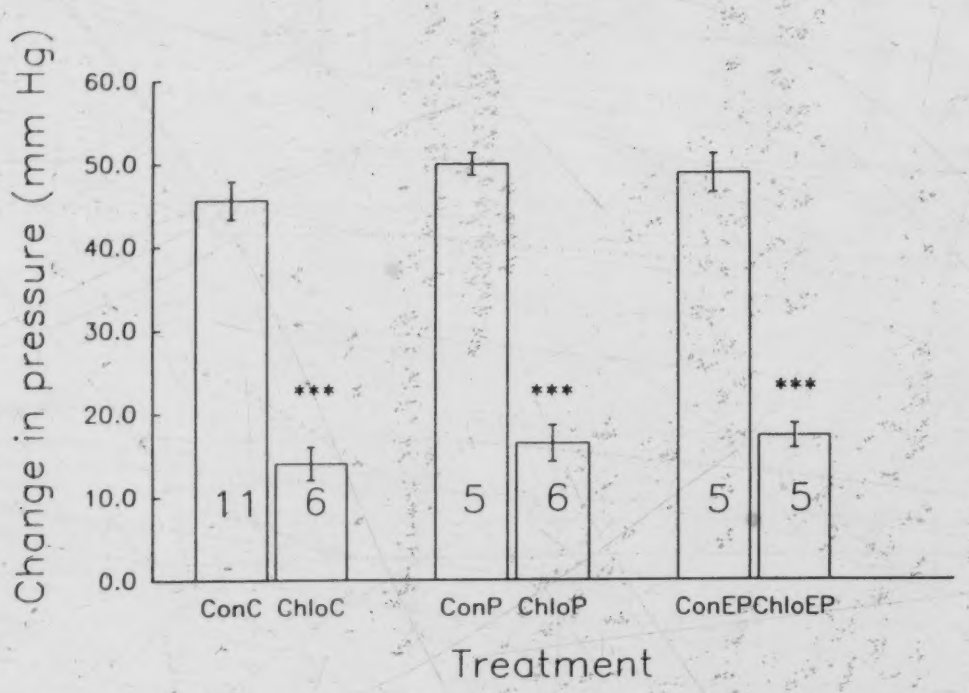

Figure 4 Comparison of the pressor responses to 100 ng AII between conscious animals and chloroformed animals. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes. (***, $p<0.005$, when compared with Conscious counterpart using Unpaired t-test) "ConC" refers to Conscious Controls, injected with saline x 10 days. "ChloC" refers to Chloroform Controls, injected with saline x 10 days. "ConP" refers to Conscious - Progesterone injected x 10 days. "ChloP" refers to Chloroform - Progesterone injected x 10 days. "ConEP" refers to Conscious - Estradiol plus Progesterone injected x 10 days. "ChloEP" refers to Chloroform - Estradiol plus Progesterone injected x 10 days.

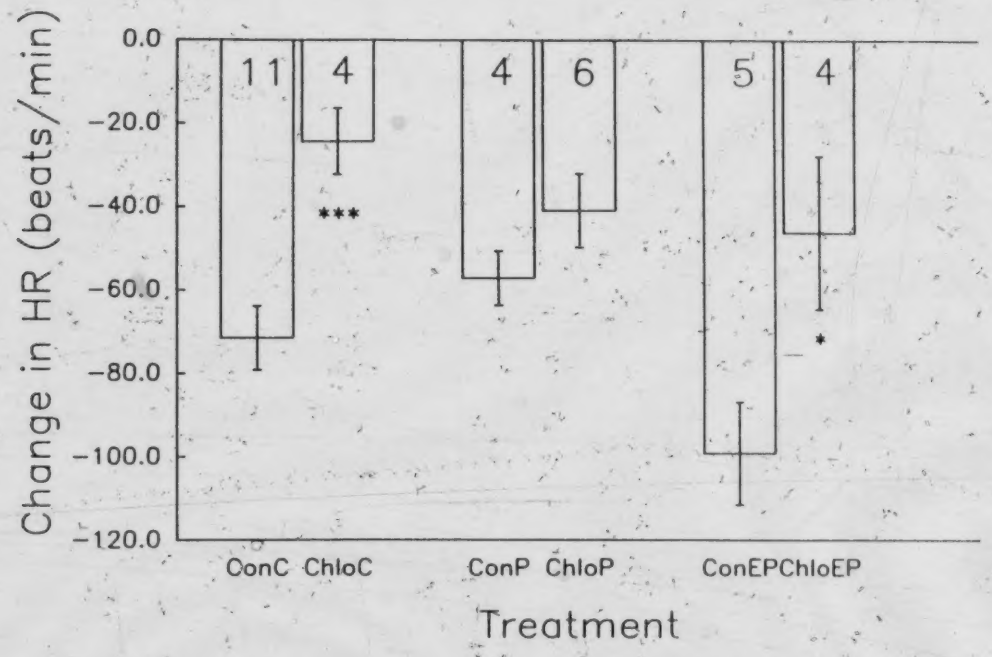

Figure 5 Comparison of changes in HR during infusion of 100ng AII between conscious animals and chloroformed animals. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes. (***, $p<0.005$; *, $p<0.05$; when compared to the conscious counterpart using Unpaired t-test). See Figure 4 for explanation of treatment abbreviations.

CHAPTER 3

EFFECTS OF PREGNANCY, ESTRADIOL AND PROGESTERONE ON ATRIAL NATRIURETIC PEPTIDE LEVELS IN THE RAT[5]

Introduction

Normal human and rat pregnancies are characterized by profound changes in fluid and electrolyte balance and blood pressure homeostasis. Despite increases in extracellular fluid and blood volume, blood pressure falls (1) and the pressor response to exogenous angiotensin II (AII) is reduced (2,3). There is increased sodium appetite (1) and increased renal sodium retention, the latter resulting primarily from increased tubular resorption (1,4). Despite this, plasma osmolality is reduced (5). One of the regulating factors in this delicate balance over hormonal control of sodium homeostasis may be atrial natriuretic peptide (ANP). This hormone, which is stored in and released from atrial myocytes in response to atrial stretch, is known to exhibit potent natriuretic, diuretic and vascular smooth muscle relaxant properties (6).

One would expect that, since pregnancy represents a state of expanded blood volume, plasma ANP levels would be higher than in the non-pregnant state, in accordance with the accompanying atrial stretch. However, reports on plasma

-5. A version of this chapter has been: submitted for publication. Novak & Kaufman 1991. American Journal of Obstetrics and Gynecology.

concentrations of ANP during normal pregnancy are conflicting. In normal human pregnancy, much of the evidence does point toward increased plasma ANP compared with the non-pregnant state. Very little evidence is available on the topic of ANP levels throughout normal rat pregnancy, and what evidence there is, is controversial. ANP has been shown to increase, to decrease, and to not change at all during rat pregnancy. This discrepancy in results could be due to differences in study design and analytical methods employed. More particularly, these differences may arise from blood collection technique, plasma extraction procedure, and/or type of assay performed (7). Another possibility could be differences in dietary sodium intake, since changes in dietary sodium intake have been shown to alter plasma concentration of ANP (8).

Much attention has been paid to changing levels of progesterone and estrogen during pregnancy. These hormones are known to increase several-fold during normal pregnancy, and have been linked to weight and water gain (9), as well as sodium homeostasis (10). Of particular interest are reports of atrial myocytes specifically accumulating estradiol, and that sex steroids may play an important role in the regulation of ANP release (11,12). One may speculate that if this is indeed the case, any condition which significantly alters sex steroid levels, such as pregnancy, has the potential to alter ANP levels as well.

We, therefore, set out to test two hypotheses; first, that ANP levels should be elevated in accordance with expanded blood volume during normal rat pregnancy, and second, that changing levels of progesterone and estrogen may alter plasma ANP levels.

Materials and Methods

The protocol for this study was approved by the University of Alberta Animal Welfare Committee (Protocol #099). All aspects of the study remained within the guidelines of this protocol.

Animals Studied:

Virgin female Long-Evans (LE) rats weighing between 200 and 225 grams were obtained from Charles River Canada, St. Foy, Quebec. They were held at least one week prior to surgery, in a temperature and humidity controlled room with 12 hours of light (0700 to 1900) and 12 hours of darkness (1900 to 0700). Surgery was not performed until the rats weighed between 240 and 280 grams. Following surgery they were caged individually and maintained on 0.3% sodium chow (Bioserv Inc., New Jersey).

The rats were surgically prepared with venous cannulae under sodium pentobarbital anesthesia (41.6 mg/kg B.wt.)[6]. Each rat was also given a standard dose of 0.2 ml atropine sulphate (0.6 mg/ml), and 0.22 ml Derapen-C (200,000 IU penicillin-G/ml) pre-operatively. One silastic cannula (0.020 in. ID, 0.037 in. OD), was non-occlusively implanted into the inferior vena cava (IVC) using the method described by Kaufman (13). This cannula was used for the collection of blood samples from conscious unrestrained animals, and

-6. A more detailed description of cannula implantation is provided in Appendix A.

allowed for repeated collections from the same animal before, during, and after pregnancy. The attrition rate of cannulation increases rapidly with advancing pregnancy, such that by day 21 of pregnancy only 10 of the initial 37 animals had functional cannulae. By 7 days post-partum, only 8 of these 10 had patent cannulae. The attrition rate of the cannulae in the ovariectomized animals was much lower. Of the initial eight animals in each group, five saline-injected, six progesterone-injected, five estradiol-plus-progesterone-injected, and all eight of the estradiol-injected animals had patent cannulae for the post-injection blood sample collection. The cannulae were exteriorized at the nape of the neck via stainless steel tubing, and filled with approximately 200 ul of heparinized saline (10,000 units/litre) to maintain patency. The animals for use in experiment B also underwent bilateral ovariectomy at this time. The animals were then allowed a minimum of 48 hours to recover from surgery and to regain their pre-operative weights before commencement of blood sampling. Body weights and general condition of each animal were monitored each day post-operative, to ensure adequate recovery and wound healing.

Experiment A:

Following collection of the first blood sample, on day 7 post-operative, vaginal smears were obtained to determine the stage of the estrous cycle. Vaginal smears were repeated every morning until proestrous was determined, at which time the female was placed with one male LE rat for a period of 36-48 hours. Day 1 of pregnancy was considered to be the first full day following initial placement together. This procedure for determining day 1 was accurate to within 12-18 hours of predicting parturition.

One ml blood samples were collected on day 7 post-operative (Pre), on days 7, 14 and 21 of pregnancy, and 7 days post-partum (Post). A group of ovariectomized females served as time controls. Blood samples were taken when their post-operative age was equivalent that of the intact/pregnant rats. A six cm extension of polyethylene tubing was connected to the stainless steel tubing at the nape of the neck. The samples were collected into dry, 1 ml tuberculin syringes and then immediately transferred into polyproplylene microcentrifuge tubes containing 2 ul of Trasylol plus 2 ul Sequester-Sol (13.8% w/v EDTA). One ml of normal saline (0.9%) was then injected through the cannula to replace the volume of blood removed from the animal. This was followed by approximately 200 ul of heparinized saline (10,000 units/litre) to fill the cannula and maintain patency over the next 7 days. Blood was immediately centrifuged at 4°C for 10 min at 1,000 g. Plasma was transferred to polypropylene storage tubes, and was then stored at -43°C until extraction.

Experiment B:

The rats were allowed 48 hours recovery from surgery. A 1.0 ml blood sample was then taken prior to commencement of hormone injections, in the same manner as described above. Hormone dose regimes were based on those used by Nakamura et al (14), with some modifications. Injections were administered subcutaneously into the loose skin of the back over a period of 10 days, one dose per day. Eight rats were injected with estradiol valerate (25 ug in 100 ul sunflower oil). Eight rats were injected with progesterone (gesterol 500 ug in 100 ul). Eight rats were injected with a combination of 25 ug estradiol plus 500 ug progesterone in 200 ul. Furthermore, eight rats given

subcutaneous injections of normal saline (100 ul per day) served as controls. These injection regimes have been proven effective by the present authors (15) in achieving plasma levels of estradiol and progesterone comparable to those seen in pregnancy. Blood samples were collected again on day 12 post-operative (following the tenth injection), in the same manner as described above.

Plasma Extraction[7]:

Sep-Pak C-18 cartridges (Fisher Scientific) were preactivated with 5 ml of 4% acetic acid in 85% ethanol, followed by 5 ml of methanol, 5 ml of distilled water, and 5 ml of 4% acetic acid in distilled water. Plasma samples were acidified by mixing 900 ul of 4% acetic acid with 300 ul of plasma. The resulting 1200 ul mixture was then drawn through the preactivated Sep-Pak C-18 column using 125 mmHg vacuum pressure. The column was then washed twice with 3 ml of distilled water. The absorbed atrial natriuretic peptide was eluted off the column and collected into polypropylene tubes using 5 ml of 4% acetic acid in 85% ethanol. Recoveries of added atriopeptin III during this extraction procedure averaged 83%. This value is comparable to that of previous reports using similar extraction procedures on Sep-Pak C-18 columns (21,22,23,).

The eluate was then placed on a drying rack and dried under an airstream. The dried extracts were stored at -43^{o}C until being resuspended in 300 ul RIA buffer on day 1 of the assay.

-7. A more detailed description of the plasma extraction procedure is contained in Appendix C.

Atrial Natriuretic Peptide Radioimmunoassay:

A double antibody radioimmunoassay was used to measure plasma atrial natriuretic peptide concentrations. All materials, except the buffer, were obtained from Peninsula Laboratories, Belmont, California.

Day 1: The buffer used to resuspend the dried sample extracts was prepared such that it contained 19 mM monobasic and 81 mM dibasic sodium phosphate (pH 7.4), 0.05 M NaCl, 0.1% BSA, 0.01% NaN_3, and 0.1% Triton X-100 (RIA buffer). This is the buffer recommended by Peninsula for use with their reagents. The dried extracts were resuspended in 300 ul of this RIA buffer and then aliquoted to 100 ul duplicates into polystyrene tubes. A standard curve was constructed using Atriopeptin III (Rat) (Peninsula Laboratories) over a concentration range of 0.05 to 125 pg/tube. Then 100 ul of rehydrated Rabbit Anti-Alpha-ANP (Rat) Serum (Lot No. 013915-8) were added to each unknown and standard tube. This antiserum recognizes the carboxy terminus of the 28-amino-acid atrial natriuretic peptide molecule. Cross-reactivities, as supplied by Peninsula Laboratories, are: Alpha-ANP (Rat); 100%, ANP (Human); 100%, ANP (8-33); 100%, Atriopeptin III (Rat); 100%, ANP (18-28); 60%, Beta-ANP (Human); 50%, Gamma-hANP; 40%, Auriculin A; 10%, ANP (Rat) (13-28); 1%, BNP; 0.001%, Atriopeptin I (Rat); 0%, Somatostatin; 0%, Oxytocin; 0%, Arg-Vasopressin; 0%. Tubes were vortexed and stored at $4^{o}C$ overnight.

Day 2: Ten uCi of ^{125}I-Atrial Natriuretic Factor, Rat, (Lot No. 022270) were resuspended in distilled water to yield 14,000 counts per minute per 100 ul. Then 100 ul of ^{125}I-ANF was added to each tube in the assay, vortexed, and incubated overnight at $4^{o}C$.

Day 3: Bound and free fractions were separated by means of normal rabbit

serum (NRS) (Lot Nos. 02179 and 021853) and goat anti-rabbit gamma-globulin (GARGG) (Lot No. 021534). Following overnight incubation, 100 ul each of NRS and GARGG were added to each standard and unknown tube in the assay. Tubes were vortexed and incubated for 2 hours at room temperature. Following the 2 hour incubation, 0.5 ml of RIA buffer were added to each tube, and the tubes were centrifuged at 4°C for 20 minutes at 2,000 g. The supernatant was then aspirated, and the precipitate was counted in a gamma counter.

Statistical Analysis:

Experiment A: All values of plasma ANP are reported as means ± standard errors of the means. Differences between groups were analyzed by Analysis of Variance (ANOVA) with the Newman-Keuls test.

Experiment B: The mean of the difference between plasma ANP before hormone-treatments (Pre) and after hormone-treatments (Post) was calculated for each group, ± standard error of the mean. Differences between groups were analyzed by ANOVA with the Newman-Keuls test. For both experiments, differences were considered significant if $p<0.05$.

The minimum detectable concentration was 0.5 pg/tube. Binding of ^{125}I-labeled ANP to the antibody was inhibited 50% by 31 pg of atriopeptin III in the first assay and 35 pg of atriopeptin III in the second assay.

To minimize any interassay variability, all atrial natriuretic peptide values reported are from samples run in two assays only. The interassay variability between these two assays was 4%.

Results

Duplicate measurements of four replicate extractions of a single plasma sample were used to calculate the intraassay coefficients of variation, which were 5% and 8% for the first and second assays, respectively. The sensitivities of the assays were 0.4 pg/tube (4.0 pg/ml) and 0.3 pg/tube (3.0 pg/ml) for the first and second assays, respectively.

Experiment A: The atrial natriuretic peptide levels obtained for all stages of pregnancy tested are shown in Figure 1. ANP levels were significantly elevated by day 7 of pregnancy ($p<0.05$) when compared to Pre-pregnant values. Levels were not significantly elevated at day 14 of pregnancy, however, there was a tendency to remain higher than the Pre-pregnant level. By day 21 of pregnancy (shortly before delivery) the ANP levels had returned to the Pre-pregnant values. Seven days post-partum (Post) the levels had risen to values which were significantly higher than both the Pre and 21 day values ($p<0.01$), Figure 1. ANP levels in the ovariectomized controls did not change significantly with time/age.

Experiment B: The mean differences in ANP levels between pre-injection and post-injection blood samples are shown in Figure 2 for the hormone- and saline-injected groups. The only group showing a significant difference compared to the saline controls was the estradiol-injected group ($p<0.05$), whose mean ANP concentration was significantly elevated.

Comment

The physiologic and/or pathologic role (if any) of ANP in pregnancy is not yet fully understood. One reason for this lies in the varied and conflicting data as far as plasma concentrations of ANP in normal human pregnancy are concerned. While some authors have reported higher plasma concentrations of ANP during normotensive human pregnancy when compared to normotensive non-pregnant controls (16,17,18), others have found no such difference (19,20). These different results are probably due to the complexity of the RIA for ANP, the different methods employed in the RIA, the blood collection, handling and storage techniques used, and the method of extraction (if any) performed (7). It is now generally agreed, that plasma extraction is a necessary step in performing an accurate RIA for plasma ANP. The majority of the reports in which extraction was performed, indicate that ANP levels do increase during normal human pregnancy.

The situation in rat pregnancy is even less clear. Few reports exist in the area of ANP levels during normal rat pregnancy and those that do, contradict one another, and are masked by major differences in blood collection source and technique, and assay type. Castro et al (21) found ANP levels to be significantly elevated on days 16-18 of normal rat pregnancy, while levels on days 10-15 and 21 of pregnancy were not significantly different from non-pregnant values. Their blood samples, however, were from an arterial source, following cervical dislocation. Jansakul et al (22) and St. Louis et al (23) both reported no increase in ANP levels during rat pregnancy, but a possible tendency for levels to be reduced near term. Both of these

investigators obtained trunk blood by decapitation, the latter also using arterial blood obtained from the abdominal aorta of rats anesthetized with sodium pentobarbital. There is no question that using trunk blood obtained following decapitation means that the sample will be a mixture of both arterial and venous blood. It has been shown that arterial ANP levels can be significantly different from venous levels in the same subject (24). Furthermore, ANP levels in plasma taken from anesthetized rats should not be compared to levels taken from conscious animals. St Louis et al (23) report ANP levels in anesthetized rat plasma to be 2.8 times lower than the ANP levels in plasma obtained by decapitation of a conscious rat.

Finally, Nadel et al (25) also reported no change in ANP levels during rat pregnancy. These investigators carefully obtained blood samples via chronic indwelling venous cannulae, 4 days following surgical implantation of the cannulae. This is the ideal method of collection according to Shaw and Weidmann (26), and is the method employed by the present authors. Unfortunately, they used a radioreceptor assay as opposed to the radioimmunoassay. This makes cross comparison between reports difficult.

The present report is the first one to use all of the suggested methods of optimizing the RIA for ANP in rat plasma during pregnancy. We were very careful to avoid as many of the "potential pitfalls" as possible (26). In brief, animals were not stressed or restrained in any way during blood collection. Blood was taken from an indwelling catheter and placed immediately in polypropylene tubes containing EDTA and Trasylol, then centrifuged rapidly to remove cells and platelets. Plasma was then stored in polypropylene tubes at -43°C. Extraction was carried out on preactivated Sep-Pak C18 octadecyl

silica cartridges. Only freshly prepared reagents and sterile water were used for both the extraction and RIA procedures. Finally, the double antibody separation of bound and free fractions was used in the RIA; this is considered to be the most reliable separation method for an RIA for ANP (26).

Prior to pregnancy, we obtained ANP levels quite similar to those of Castro et al (21) who used a similar extraction procedure and the same RIA procedure as we did. Our results indicate, however, that ANP levels are elevated by day 7 of normal rat pregnancy. This contradicts the findings of Castro et al (21) who did not find a significant rise in ANP levels until days 16-18 of pregnancy. Our observation, that ANP levels were elevated by day 7, was as expected, in accordance with the volume expansion known to occur during this stage of pregnancy (1). It may also partly explain the reduced vascular reactivity to angiotensin II known to occur at this time (2,3,15). Our finding, that plasma ANP levels returned to non-pregnant values near term, is in agreement with Castro et al (21), but was unexpected since blood volume expansion (1) and reduced vascular reactivity to AII are maximal at this time (3,15). It may, however, explain the avid sodium retention known to occur near term gestation in the rat (4).

It is generally agreed that post-partum levels of ANP are significantly elevated in both the human and the rat (18,21,22,25). Our results are in agreement with these findings. The post-partum rise in ANP suggests that ANP is undoubtedly involved in the mobilization and ultimate excretion of the excess maternal sodium and water occurring in the first few days post-partum (27).

Our results indicate that in the early stages of normal rat pregnancy, the

increased blood volume and accompanying atrial stretch stimulates higher plasma levels of ANP. By the third week of normal rat pregnancy, however, the situation changes. Several possibilities have been put forth to explain this situation: somehow, atrial volume receptors reset themselves and are no longer responding to intravascular volume changes, or, the elevated ANP secretion in pregnancy is balanced by an increased rate of clearance so that circulating levels are unchanged, or, acute volume changes, not chronic changes are the required stimulus for heightened ANP secretion (25). We would like to put forth another possibility: that the plasma levels of estradiol and progesterone, and the ratio of estradiol to progesterone in the plasma play a regulatory role in the secretion of ANP during normal rat pregnancy.

Our results indicate that in the presence of progesterone alone, ANP levels tend to rise, but are not significantly higher compared to ovariectomized control levels. In the presence of estradiol alone, ANP levels were significantly elevated compared to control levels. It has been shown that estradiol is specifically taken up and concentrated in the nuclei of ANP-containing atrial myocytes (12). Therefore, this may suggest a regulatory role for estradiol on ANP secretion. Finally, when both estradiol and progesterone were injected, ANP returned to levels very similar to that of the ovariectomized controls. These data again support the hypothesis of Back, Forssmann and Stumpf (12), that sex steroids play a role in the regulation of ANP release from cardiac myocytes.

Another interesting relationship was noted during the course of this investigation. During normal human pregnancy, both progesterone and estradiol levels are known to increase gradually to term (28). As mentioned previously,

this also appears to be the case for ANP during normal human pregnancy, ie: ANP levels rise gradually to term. During normal rat pregnancy, however, progesterone rises to a peak around mid-gestation, then falls back down to non-pregnant values near term (29), while estradiol levels gradually increase to term (30). In summary then, it is known that the balance of estradiol and progesterone is different in human pregnancy versus rat pregnancy, particularly in the third trimester. Of particular interest then, is our finding that the pattern of changes in ANP levels is different during rat pregnancy versus human pregnancy in the third trimester. This difference in ANP levels may lie in the different balances of sex steroids between species. This further supports the hypothesis that sex steroids may play a role in the regulation of ANP release, especially during pregnancy.

In summary, we have determined that ANP levels are significantly elevated by day 7 of normal rat pregnancy, but then return to non-pregnant levels in the third week of gestation. This is different from the human condition in which a gradual rise in ANP levels is seen throughout gestation to term. ANP levels post-partum are significantly elevated in both the rat and human. Treatments with exogenous progesterone, and a combination of estradiol plus progesterone had no effect on plasma ANP levels in acyclic rats, while estradiol treatments caused a significant increase in plasma ANP levels. The balance of estradiol and progesterone is different during human pregnancy versus rat pregnancy, and may be the reason for the difference in ANP levels during human and rat pregnancies, particularly in the third trimesters. This suggests that sex steroids, more particularly the balance of progesterone and estradiol, may play a role in the regulation of plasma ANP levels during pregnancy.

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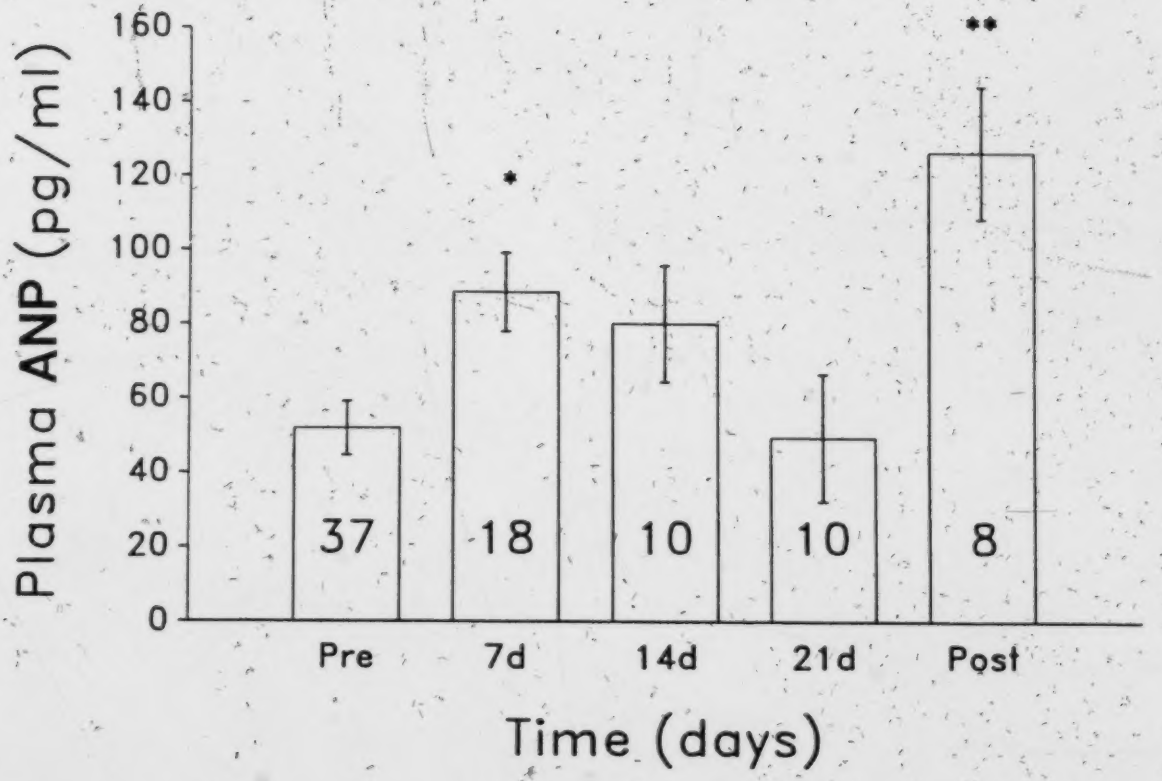

Figure 1: ANP levels obtained prior to pregnancy (Pre), at days 7, 14 and 21 of pregnancy and 7 days post-partum (Post) in the rat. Time refers to stage of pregnancy. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes. (*, $p<0.05$; **, $p<0.01$; when compared to Pre-pregnancy using Newman-Keuls Test).

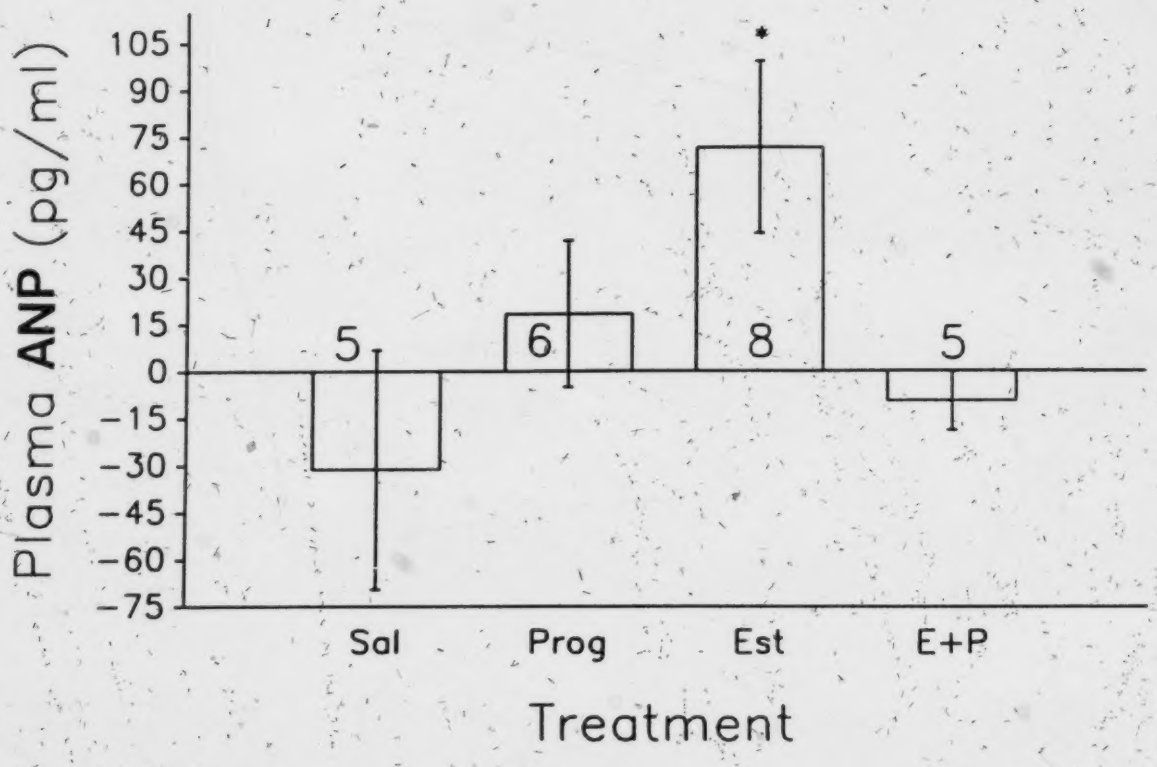

Figure 2: The mean differences in ANP levels between pre-injection and post-injection blood samples. Treatment refers to the following: "Sal" refers to the saline-injected controls. "Prog" refers to the progesterone-injected animals. "Est" refers to the 25 ug estradiol-injected animals. "E+P" refers to the 25 ug estradiol plus 500 ug progesterone-injected animals. The vertical bars delineate standard errors of the means. The numbers within the bars delineate sample sizes. (*, $p<0.05$, when compared to the saline-injected controls using Newman-Keuls Test).

CHAPTER 4

GENERAL DISCUSSION AND CONCLUSION

It is now well established that a decrease in pressor responsiveness to exogenous AII develops during normal human pregnancy as early as the 10th week of gestation (1), ie: the phenomenon is readily demonstrable in the first trimester of human pregnancy. The decrease in pressor responsiveness has been noted to occur in several pregnant animal models (2,3,4,5,6,7), however, in no case has the refractoriness been demonstrated during the first trimester. This is a characteristic that is important to establish in a potential model of human pregnancy. Furthermore, there is very little information published regarding the role of the heart rate (baroreceptor) reflex in this decreased responsiveness to AII during pregnancy.

There are obvious ethical and practical reasons for the establishment of a pregnant animal model which manifests similar changes in cardiovascular parameters to those observed in pregnant humans. Establishment of such a model would certainly facilitate research of the mechanisms involved in both normal and pathological states.

The first aim of this study was to establish the rat as a valid model for human pregnancy by confirming that the pressor response to injected AII does, indeed, fall in conscious, unrestrained pregnant rats within the first trimester of pregnancy. The use of conscious, chronically instrumented animals is of importance since pregnant animals are particularly susceptible to surgical trauma and the effects of anesthesia. This is of further importance

in the case of AII as it becomes critically involved in modulating cardiovascular function in a stressed animal, and assumes a major controlling role which it would not normally have (8).

This is the first study to show a significant reduction in pressor response by as early as the seventh day, ie. first trimester, of animal pregnancy. This is an important finding because it better compares to the human condition. We also found a reduction in resting BP by the last week of pregnancy in the rat. This is in agreement with the normal pregnant human condition (9). Based on these findings it is concluded that the rat is a good candidate as a model for the study of altered vascular reactivity to exogenous AII during human pregnancy.

Of late, there has been much attention paid to the involvement of estrogens and progestins in the mechanism of decreased pressor responsiveness to vasopressors during pregnancy. Much controversy exists in the literature regarding the role of these sex steroids in the decreased pressor responsiveness to AII during pregnancy. It is felt that this discrepancy exists because of the vastly different conditions under which the results were obtained.

The second aim of the present study was to try to resolve the controversy over the possible effects of estrogen and progesterone on the pressor response to AII by carefully performing the experiments in conscious, unrestrained animals, remote from the effects of anesthesia and surgery.

We found that estradiol treatments did not significantly alter the pressor response to exogenous AII. This agrees with the findings of Chesley & Tepper (10) for conscious pregnant women. We also found that neither progesterone nor the estradiol-progesterone combination significantly affected the pressor

response to AII. Although this agrees with the findings of Chesley & Tepper (10), it differs from the results obtained by Nakamura et al (11) who used chloroform-anesthetized rats.

Since our experiments were carefully conducted in conscious, unrestrained animals remote from surgery and anesthesia, we believe our results to be sound and to provide a clearer indication of the normal physiological situation than any of the previous experiments where anesthetized, recently anesthetized, and/or restrained animals were used.

The third aim of this study was to investigate whether there is a change in the heart rate reflex accompanying the pressor response to AII during pregnancy, and whether the heart rate response is at all affected by estradiol or progesterone. Very little information is available on this subject at present, and what is available is clouded by the use of acute animal preparations, anesthesia and animal restraint during testing.

We observed no change in resting heart rate during pregnancy, despite the significant reduction in resting BP observed at 21 days of pregnancy.

The HR response to exogenous AII infusion was, however, attenuated during pregnancy. The reflex reduction in HR tended to be less robust as early as 7 days of pregnancy. This suggests the beginnings of an impairment of the baroreceptor reflex. Although by day 14, values had somewhat reset themselves closer to the non-pregnant state, and by day 21, the heart rate response was completely lost. This was despite the 20 mmHg increase in BP. Treatments with either estradiol, progesterone, or both had no effect on the heart rate reflex to AII.

We conclude, therefore, that the baroreceptor reflex, in response to exogenous AII, is attenuated by day 21 of pregnancy in the Long-Evans rat. This agrees with the results of Ismay, Lumbers and Stevens (12) who performed their study in conscious pregnant ewes. It appears from the results of our study that something is interfering with both the pressor response and baroreceptor reflex in pregnancy.

Attention was then turned toward atrial natriuretic peptide (ANP). In view of its established renal and cardiovascular (hypotensive) actions, ANP is an obvious candidate in the search for the mechanism of pregnancy-induced changes in blood pressure control and fluid homeostasis. However, the physiologic and/or pathologic role (if any) of ANP in pregnancy is not yet fully understood.

It is now generally agreed, that plasma ANP levels do increase during normal human pregnancy (13,14,15). The situation in rat pregnancy, however, is less clear. Few reports exist in the area of ANP levels during normal rat pregnancy and those that do, contradict one another, and are masked by major differences in blood collection source and technique, and assay type (16,17,18,19).

The present results indicate that ANP levels are, indeed, elevated by day 7 of normal rat pregnancy. This rise in ANP levels is as expected, in accordance with the volume expansion known to occur during pregnancy (20), and may partly explain the reduced vascular reactivity to angiotensin II known to occur at this time. Our finding, that plasma ANP levels returned to non-pregnant values near term was unexpected since blood volume expansion (20) and reduced vascular reactivity to AII are maximal at this time (7). It may, however, explain the avid sodium retention known to occur near term gestation in the rat (21).

It is generally agreed that post-partum levels of ANP are significantly elevated in both the human and the rat (15,16,17,19). Our results are in agreement with these findings. The post-partum rise in ANP suggests that ANP is undoubtedly involved in the mobilization and ultimate excretion of the excess maternal sodium and water occurring in the first few days post-partum (22).

Our results indicate that in the early stages of normal rat pregnancy, the increased blood volume and accompanying atrial stretch stimulates higher plasma levels of ANP. By the third week of normal rat pregnancy, however, the situation changes. Several possibilities have been put forth to explain this situation: somehow, atrial volume receptors reset themselves and are no longer responding to intravascular volume changes, or, the elevated ANP secretion in pregnancy is balanced by an increased rate of clearance so that circulating levels are unchanged, or, acute volume changes, not chronic changes are the required stimulus for heightened ANP secretion (19). We would like to put forth another possibility, which led to the final aim of this study: that the plasma levels of estradiol and progesterone, and the ratio of estradiol to progesterone in the plasma play a regulatory role in the secretion of ANP during normal rat pregnancy.

Our results indicate that in the presence of estradiol alone, ANP levels were significantly elevated compared to control levels. It has been shown that estradiol is specifically taken up and concentrated in the nuclei of ANP-containing atrial myocytes (23), suggesting a regulatory role for estradiol on ANP secretion (23). In the presence of progesterone alone, ANP levels tend to rise, but are not significantly higher compared to ovariectomized control levels. Finally, when both estradiol and progesterone were injected, ANP

returned to levels very similar to that of the ovariectomized controls. These data again supports the hypothesis that the sex steroids interact in some manner to influence ANP release from cardiac myocytes (23).

Another interesting relationship was noted during the course of this investigation. During normal human pregnancy, both progesterone and estradiol levels are known to increase gradually to term (24); this is associated with a steady increase in ANF levels to term. During normal rat pregnancy, however, progesterone rises to a peak around mid-gestation, then falls back down to non-pregnant values near term (25), while estradiol levels gradually increase to term (26); this is associated with an initial increase in plasma ANP levels and a fall just before parturition.

It is probable that the important factor influencing ANP secretion is not the absolute concentrations of estrogen or progesterone, but rather the relative plasma concentrationsof the two hormones. Thus, the relative levels characteristic of human pregnancy and early rat pregnancy cause increased ANP levels, while the change in this pattern at the end of the rat pregnancy, causes ANP levels to fall.

The primary determinant of the reduced pressor response to AII during pregnancy remains open to question. Very little attention has been given to the involvement of the baroreceptors in the cardiovascular response to exogenous vasoconstrictors during pregnancy. Some interesting aspects of the heart rate response have been presented in this study, and we feel that more careful consideration is needed on this integral component in the future. Furthermore, the idea that sex steroids may play a role in regulating ANF levels is a new one, and more attention needs to be focussed on this topic in the future.

Despite conflicting evidence, the prostaglandins (PG) remain strong contenders in the search for mediators of the physiological changes observed during pregnancy. Moreover, PG's are known to be important regulators of cardiovascular and renal physiology. They have potent effects on vascular reactivity, renal blood flow, and the handling of salt and water by the kidney (27).

Prostaglandin E_2 (PGE) and prostacyclin (PGI) are both potent vasodilators. In addition, PGI is an inhibitor of both platelet aggregation and uterine vessel contractility (28). These effects favor the prevention of maternal hypertension and other manifestations of pre-eclampsia. In other words, they favor the normal pregnant state.

Prostaglandin F_2 (PGF) and thromboxane (another metabolite of arachadonic acid similar to PG's) exert opposing effects to those of PGE and PGI. They are potent vasoconstrictors, and thromboxane stimulates platelet aggregation and uterine vessel contractility (28). These effects, if left unchecked, would lead to pre-eclamptic manifestations.

It has been shown that during normal pregnancy there is increased production of PGI, PGE and thromboxane (28). Indeed, several reports provide convincing evidence that vasodilatory PG's do play a role in the reduced pressor responsiveness to AII (and other vasoconstrictors) during pregnancy (6,29,30,31). These investigators all found that treatments with PG synthetase inhibitors could normalize the pressor response to AII during pregnancy. The report of Conrad & Colpoys (32) contradicts these findings. They found that PG synthesis inhibition did not restore the pressor response in pregnant rats to non-pregnant levels. The reason for this difference in results is unclear since Conrad & Colpoys (32) used a similar method to the other investigators.

Further studies are required to determine the reason for this discrepancy.

Prostaglandins have also been identified as the cause of reduced pressor responses to AII in many non-pregnant situations (31,33). For example, animals on high protein diets and animals infused with parathyroid hormone exhibit blunted pressor responses secondary to elevated PG levels (31).

Considerable evidence now indicates that pre-eclampsia is associated with an imbalance of increased thromboxane production and deficient PGI production (28,34). It has been suggested that this deficiency in vasodilatory PG's, which would normally lessen the effects of thromboxane, is responsible for the hypertension, increased vascular sensitivity to AII, and uterine vessel constriction known to occur in pre-eclampsia (34).

One cannot ignore the proven clinical ability of aspirin to reduce the incidence of pre-eclampsia in high risk patients (28). Aspirin is known to selectively inhibit the production of thromboxane while leaving PGI levels unchanged (28). Therefore, aspirin appears to correct the imbalance of excess thromboxane in pre-eclampsia, thereby allowing PGI to exert its vasodilatory effects as it would in a normal pregnancy. This results in a lowering of blood pressure and a decrease in the severity of other pre-eclamptic manifestations.

The present study has ruled out estradiol and progesterone as the direct cause of reduced responsiveness to AII. ANP may be partly responsible for the vascular refractoriness in the early stages of rat pregnancy, but certainly not in the third trimester. At this time, vasodilatory PG's appear to be the most convincing candidate for the cause of the vascular refractoriness to AII during pregnancy.

In summary, it is concluded that:

1) Resting blood pressure is significantly reduced by the third week of pregnancy in the Long-Evans rat,

2) The pressor response to exogenous AII is attenuated as early as the seventh day of pregnancy in the rat. Thus, the pregnant rat represents a valid model for the study of altered vascular reactivity to exogenous AII in the pregnant human,

3) The baroreceptor reflex is significantly attenuated by day 21 of pregnancy,

4) Neither estradiol, nor progesterone, nor a combination of estradiol and progesterone is responsible for the attenuated pressor response, and/or heart rate response, to exogenous AII observed during pregnancy.

5) ANP levels are significantly elevated by day 7 of normal rat pregnancy, but then return to non-pregnant levels in the third week of gestation. This is different from the human condition in which a gradual rise in ANP levels is seen throughout gestation to term.

6) ANP may contribute to the reduced pressor response to AII in early pregnancy, but is unlikely to play a role near term.

7) ANP levels post-partum are significantly elevated in the rat. This is in agreement with the human condition.

8) Treatments with exogenous progesterone, and a combination of estradiol plus progesterone had no effect on plasma ANP levels in acyclic rats, while estradiol treatments caused a significant increase in plasma ANP levels.

9) Sex steroids, more particularly the balance of progesterone and estradiol, may play a role in the regulation of plasma ANP levels during

pregnancy. The balance of estradiol and progesterone in different during human pregnancy versus rat pregnancy, and may be the reason for the difference in ANP levels during human and rat pregnancies, particularly in the third trimesters.

The present study has laid some of the groundwork for the basic hormonal and cardiovascular changes that occur in the rat during pregnancy. It is felt that this groundwork has put us one step closer to understanding blood pressure and endocrine regulation in normal pregnancy, and therefore, one step closer to understanding and treating blood pressure and endocrine abnormalities in pregnancy.

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APPENDIX A

Surgical Procedure for implantation of chronic venous and arterial cannulae:

Each rat was given an intraperitoneal injection of sodium pentobarbital (Somnotol) for anesthesia (41.6 mg/kg B.wt). This dosage was modified for the female rats, as it was noted that the females tended to be more susceptible to the effects of anesthesia than the males. The females fell into a deeper state of anesthesia than the males, and became hypothermic when given the regular dose (57.5 mg/kg B.wt). Many of them did not wake up from the anesthetic. Consequently, the dose was lowered by approximately one-third. Each rat was also given a standard dose of 0.2 ml atropine sulphate (0.6 mg/ml) intraperitoneally, to minimize pulmonary congestion, and 0.22 ml Derapen-C (200,000 IU penicillin-G/ml) intramuscularly, to resist infection.

The nape of the neck and abdomen were then shaved and prepared with betadine to improve sterility during surgery. The surgical area itself was first cleaned with soap and water, followed by Virocidin-X (5% dimethylethanol, 0.5% sodium hypochlorite, 0.01% sodium dodecyl sulphate). The animal was placed in dorsal recumbency and all four limbs were restrained. A surgical drape was then placed over the animal leaving only the shaved abdominal region exposed. All surgical instruments were pre-soaked in Hibidil (0.05% w/v chlorhexidine gluconate in aqueous solution with 4% v/v isopropyl alcohol) to further improve sterility of the procedure. The surgeon then donned a gown, mask and sterile surgical gloves. All cannulae were prepared in advance, gas-sterilized, then stored in sterile packages until required.

A 5 cm midline skin incision was made using a sterile No. 15 scalpel blade. The linea alba was located and lifted using Adson forceps, a puncture wound was then made through the linea alba using the scalpel. Metzenbaum scissors were then used to enlarge the muscle wall incision. An abdominal retractor was placed to retract the left abdominal wall. The cecum, large and small intestines were then removed from the abdominal cavity, wrapped in saline soaked 4x4 inch gauze dressings, and laid to the animals' right hand side. This exposed the uterine horns, ovaries, inferior vena cava (IVC), abdominal aorta, renal vessels and left kidney. The ureter was located and care was taken to avoid trauma to this delicate structure.

For the control animals, bilateral ovariectomy was performed at this time by ligating both the blood supply and the tip of the uterine horn with 4-0 prolene. The ovaries were then cut away using Metzenbaum scissors.

The abdominal aortic cannula was inserted first according to the following procedure. The connective tissue was dissected away to expose the abdominal aorta and IVC. A 1.5 cm length of the abdominal aorta, between the renal arteries and the bifurcation, was cleared and separated from the adjacent IVC by blunt dissection. A 6-0 prolene anchoring suture was placed in the tunica adventitia immediately caudal to the proposed site of cannula insertion. Two more 4-0 silk anchoring sutures were placed into the left psoas muscle, lateral and slightly craniad to the proposed site of insertion. A microvascular clamp was then placed on the abdominal aorta 1 cm craniad to the proposed site of insertion. Care was taken to avoid clamping the renal arteries, and the clamp was never left on longer than 1 minute at a time to ensure adequate blood supply was maintained to the lower body. Iris forceps were used to grasp the artery, and a puncture wound was made using a 27-gauge needle.

PE10 cannula, previously prepared with a U-shaped "crook" and a cuff near the insertion end, was then inserted into the aorta and advanced craniad until the base of the "U" lay at the puncture site. The bulldog clamp was then removed. The 6-0 prolene was used to lash the base of the "U" to the aorta and the 4-0 silk sutures were used to lash the cannula to the psoas muscle, with one suture above and one below the cuff. This unique shape and anchoring procedure prevents the pressure of the blood within the aorta from forcing the cannula out of the vessel. The cannula was then anchored to the abdominal wall near the incision using 4-0 silk.

Attention was then turned to placement of the IVC cannulae. The digestive organs already wrapped in gauze were moved over to the animal's left side, and the abdominal retractor was moved to retract the right abdominal wall. This exposed the IVC and right kidney. The connective tissue over a 1.5 cm length of the IVC was removed by blunt dissection. Four 4-0 silk anchoring sutures were placed in the psoas muscle, lateral and caudad to the proposed sites of cannulae insertion. Iris forceps were used to grasp the wall of the IVC, and a puncture wound was made using a 23-gauge needle. Blood loss was minimized by lifting up on the wall of the artery to create negative pressure. A silastic cannula (0.02 in. ID, 0.037 in. OD) previously prepared with a cuff and beveled end was then inserted and advanced approximately 3 cm craniad. It was then lashed down to the psoas muscle using the 4-0 silk ligatures, one above and one below the cuff. A second puncture wound was then made approximately 1 cm craniad to the first, using a 27-gauge needle. A PE10 cannulae, previously prepared with a cuff, was then inserted and advanced approximately 3 cm craniad. This cannula was also lashed down in the same manner as the first.

Both cannulae were anchored to the muscle wall using 4-0 silk. The abdominal contents were then replaced, taking care not to tangle with the cannulae. The muscle wall was closed using continuous-locking sutures in 3-0 chromic, allowing the cannulae to exit between ligatures. The skin was then approximated in 3-0 silk using interrupted sutures, allowing the cannulae to exit between the ligatures. The ends of these ties were trimmed short to deter the animal from chewing them out prematurely. (The skin sutures did not need to be removed, as the animal would remove them on its own after approximately 10 days. Only occasionally would an animal remove them prematurely, in which case the incision would be repaired under Halothane anesthesia.)

At this point the surgical drape and limb restraints were removed, and the animal was turned over into the prone position to expose the nape of the neck. A 1.5 cm lateral skin incision was made approximately 1 cm caudad to the ears. Blunt dissection was carried out in all directions under the skin in order to make room for the pedestal apparatus and cannulae. Each cannula was then fed subcutaneously from the abdominal incision to the neck incision using a blunt probe. Care was taken to avoid kinking or twisting the cannulae during this procedure. The cannulae were then hooked up to "L"-shaped pieces of stainless steel tubing anchored with dental acrylic to a pedestal made from polypropylene monofilament sheeting. The other ends of the steel tubing were brought through the skin via puncture wounds located between the ears. These ends were turned upright, so that the pedestal (base of the "L") lay flat against the muscle wall. Each cannula was anchored to the steel tubing a 4-0 prolene ligature, and the neck incision was then approximated using interrupted sutures in 4-0 prolene.

Each cannula was then checked for patency, flushed, and filled with approximately 200 ul of heparinized saline (10,000 units/litre), to maintain patency, then sealed with silastic caps. The aortic cannula also contained approximately 20 ul of polyvinylpyrrolidone solution (8 mg PVP/10 ml heparinized saline) to prevent the arterial blood from forcing its way into the cannula and coagulating. The cannulae did not require daily flushing to maintain patency, and infection was rarely a problem. They were left untouched for up to 7 days at a time without affecting patency, however, the attrition rate increased greatly with advancing pregnancy. The expanding abdominal girth placed stress on the cannulae and often pulled them out of the vessels, or off the steel tubing at the neck. This was only somewhat compensated for by leaving longer loops of slack within the abdomen and in the neck area.

With practice, the surgical procedure can be completed in approximately one hour. However, only about one in every thirty rats which underwent this surgery had patent arterial cannulae at 21 days of pregnancy.

To the best of our knowledge, this is the longest that chronic cannulae, particularly arterial, have ever been reported to be maintained. I feel that, despite the frustrations of having only one in thirty succeed to term, this was the ideal way to carry out such an experiment in that each animal is, effectively, its own control; ie. repetitive sampling from the same conscious, unrestrained animal.

APPENDIX B

ADDITIONAL FIGURES FOR CHAPTER 2

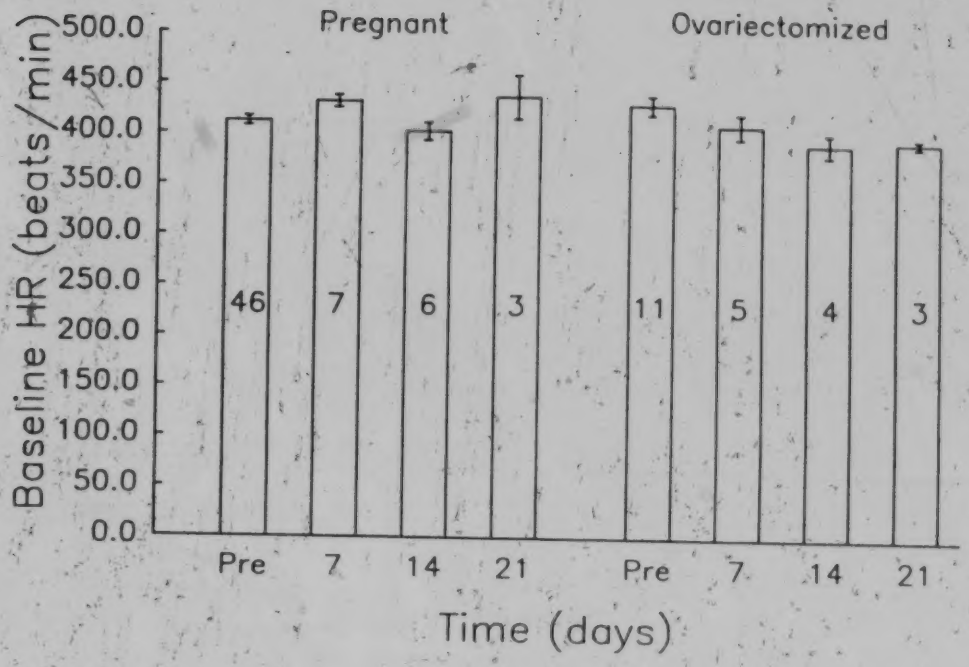

Figure 1 Resting heart rates of pregnant and acyclic female rats. Time refers to stage of pregnancy. The ovariectomized rats were tested when their post-operative ages were equivalent to that of the pregnant rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes.

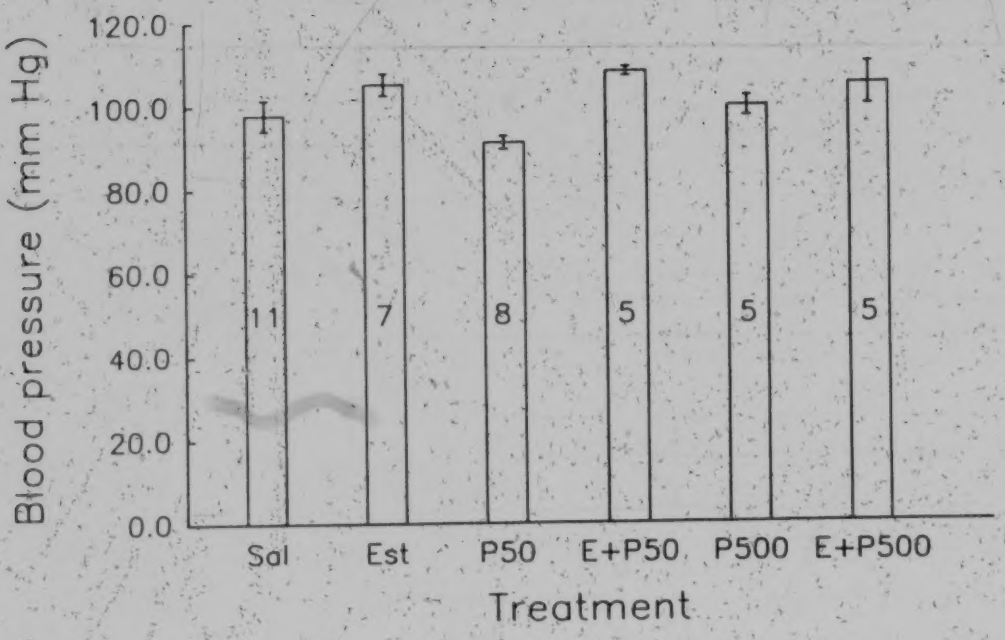

Figure 2 Resting arterial blood pressure of steroid-treated, acyclic female rats. "Sal" refers to the saline-injected controls. "Est" refers to the estradiol-injected rats. "P50" refers to the 50ug progesterone-injected rats. "E+P50" refers to the 50ug progesterone plus estradiol combination-injected rats. "P500" refers to the 500ug progesterone-injected rats. "E+P500" refers to the 500ug progesterone plus estradiol combination-injected rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes.

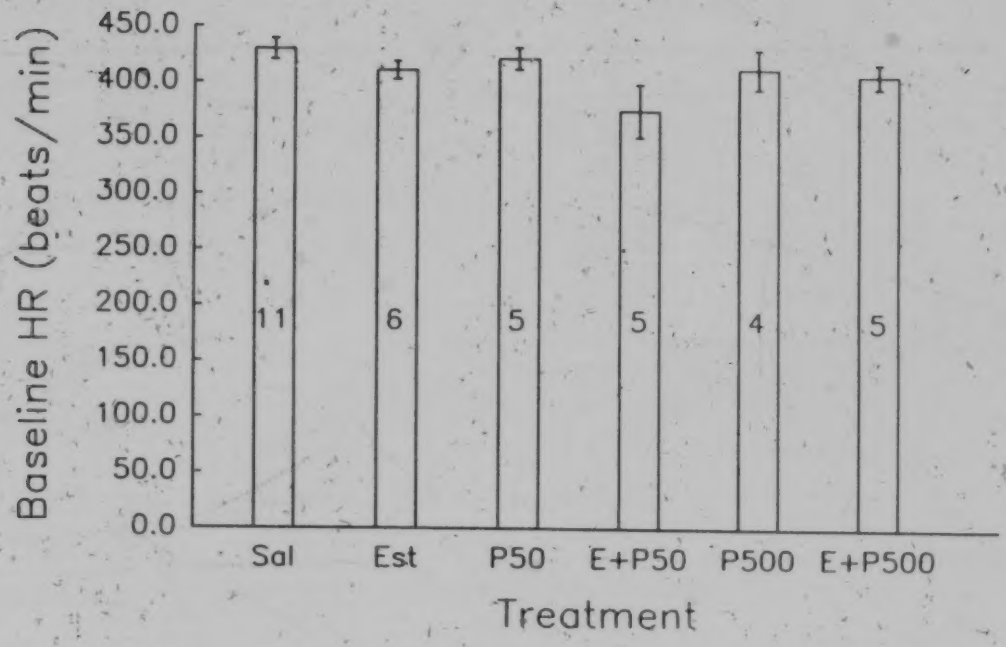

Figure 3 Resting heart rates of steroid-treated, acyclic female rats. "Sal" refers to the saline-injected controls. "Est" refers to the estradiol-injected rats. "P50" refers to the 50ug progesterone-injected rats. "E+P50" refers to the 50ug progesterone plus estradiol combination-injected rats. "P500" refers to the 500ug progesterone-injected rats. "E+P500" refers to the 500ug progesterone plus estradiol combination-injected rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes.

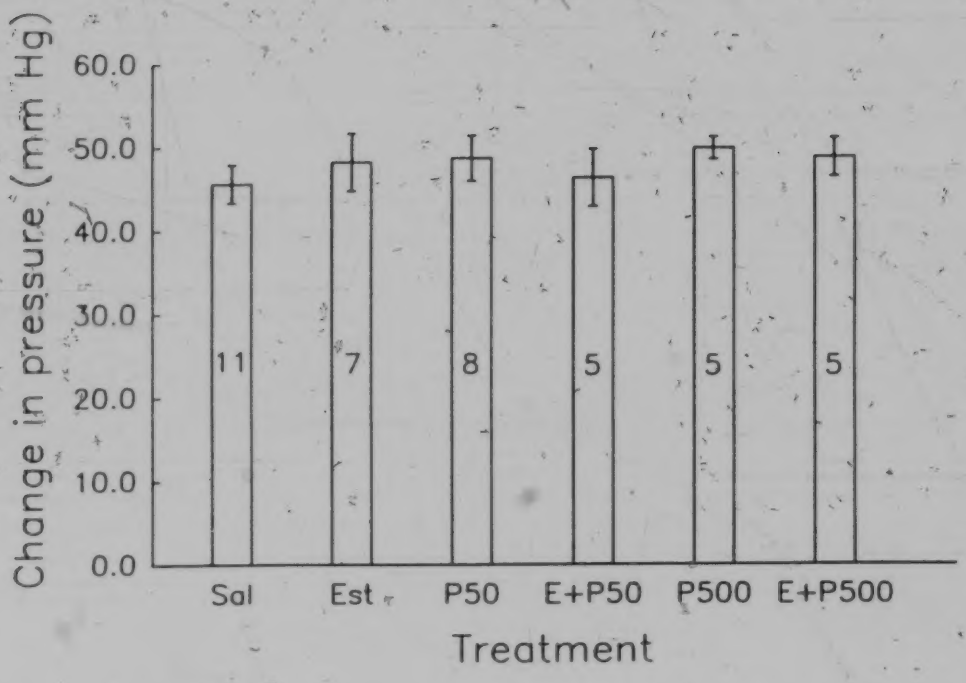

Figure 4 Pressor responses to injected AII (100 ng in 10 ul) in steroid-treated, acyclic female rats. "Sal" refers to the saline-injected controls. "Est" refers to the estradiol-injected rats. "P50" refers to the 50ug progesterone-injected rats. "E+P50" refers to the 50ug progesterone plus estradiol combination-injected rats. "P500" refers to the 500ug progesterone-injected rats. "E+P500" refers to the 500ug progesterone plus estradiol combination-injected rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes.

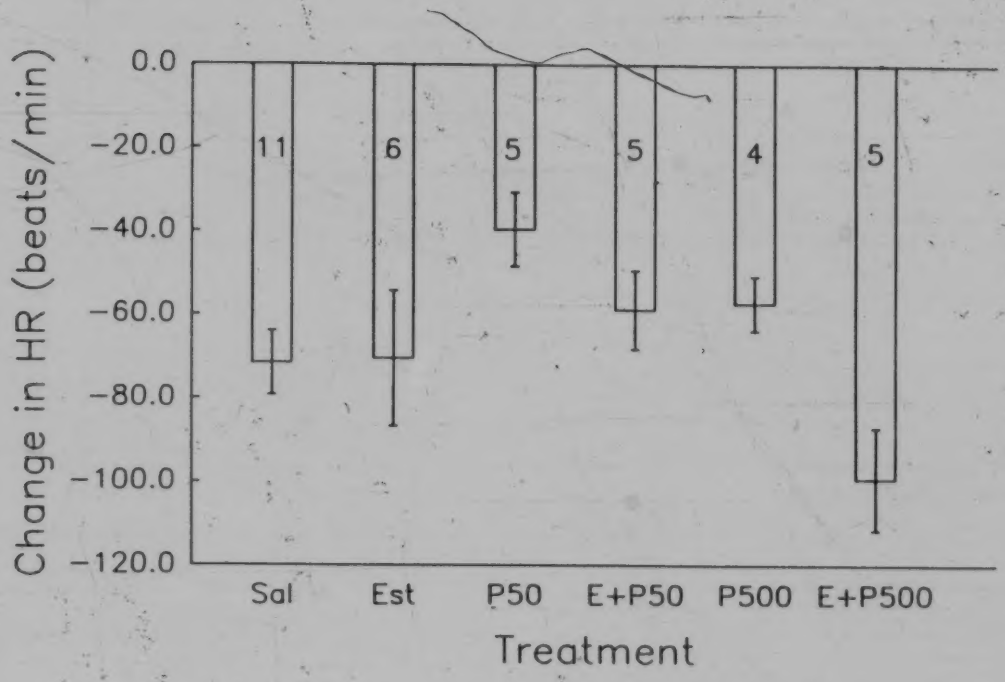

Figure 5 Heart rate responses to injected AII (100 ng in 10 ul) in steroid-treated, acyclic female rats. "Sal" refers to the saline-injected controls. "Est" refers to the estradiol-injected rats. "P50" refers to the 50ug progesterone-injected rats. "E+P50" refers to the 50ug progesterone plus estradiol combination-injected rats. "P500" refers to the 500ug progesterone-injected rats. "E+P500" refers to the 500ug progesterone plus estradiol combination-injected rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes. No significant differences noted when compared to the saline injected controls using Newman-Keuls test.

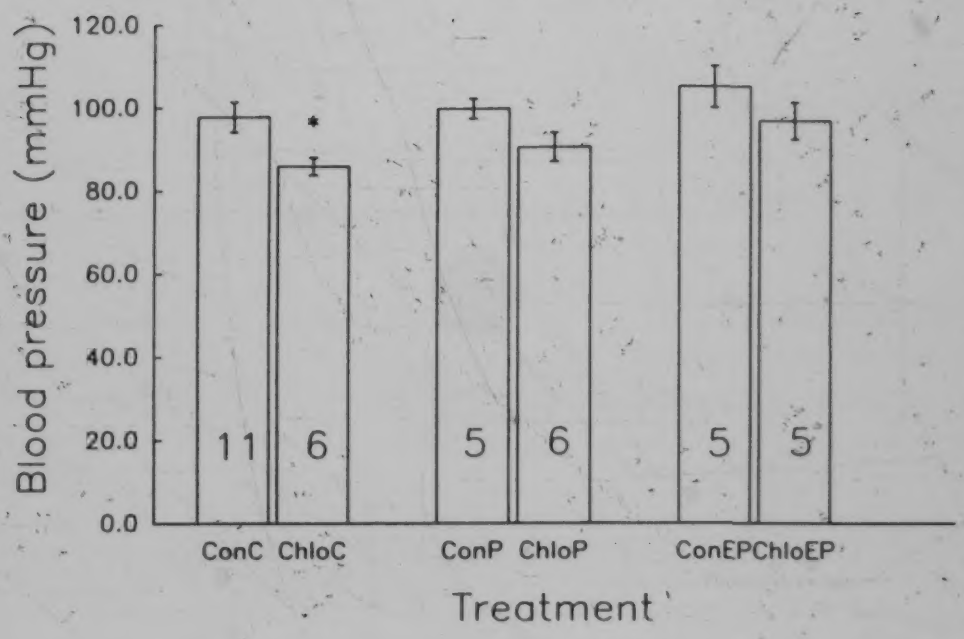

Figure 6 Comparison of the resting blood pressures between conscious and chloroformed rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes. (*, $p<0.05$, when compared with conscious counterpart using Unpaired t-test). "ConC" refers to Conscious Controls, injected with saline x 10 days. "ChloC" refers to Chloroform Controls, injected with saline x 10 days. "ConP" refers to Conscious - Progesterone injected x 10 days. "ChloP" refers to Chloroform - Progesterone injected x 10 days. "ConEP" refers to Conscious - Estradiol plus Progesterone injected x 10 days. "ChloEP" refers to Chloroform - Estradiol plus Progesterone injected x 10 days.

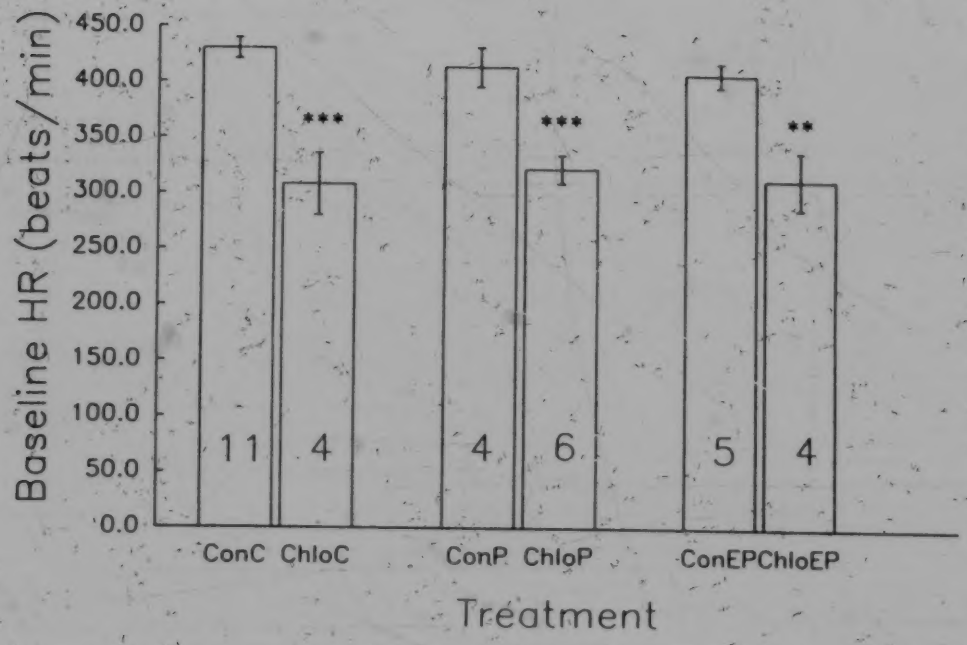

Figure 7 Comparison of resting heart rates between conscious and chloroformed rats. The vertical bars delineate standard errors of the means. The numbers within the bars indicate sample sizes. (**, $p<0.01$; ***, p,0.005; when compared with conscious counterpart using Unpaired t-test). "ConC" refers to Conscious Controls, injected with saline x 10 days. "ChloC" refers to Chloroform Controls, injected with saline x 10 days. "ConP" refers to Conscious - Progesterone injected x 10 days. "ChloP" refers to Chloroform - Progesterone injected x 10 days. "ConEP" refers to Conscious - Estradiol plus Progesterone injected x 10 days. "ChloEP" refers to Chloroform - Estradiol plus Progesterone injected x 10 days.

APPENDIX C

STANDARD EXTRACTION PROCEDURE

(this was the method used to obtain the results for Chapter 3)

Preparation of Plasma Samples:

-ratio of 1.0 ml plasma to 3 ml 4% acetic acid in sterile water

Extraction using Sep-Pak C-18 Columns (Fisher Scientific):

Column Pre-activation, steps 1-4

1) 5 ml 4% acetic acid in 85% ETOH
2) 5 ml Methanol
3) 5 ml H_2O (sterile or fresh distilled)
4) 5 ml 4% acetic acid in H_2O (sterile or fresh distilled)
5) Add plasma solution
6) 2 x 3 ml H_2O wash (sterile or fresh distilled)
7) Collect the eluate with 3 ml 4% acetic acid in 85% ETOH
8) Dry under air stream in polypropylene tubes
9) Cover with parafilm, and freeze until ready to perform RIA

While trying to establish the above protocol, numerous observations were made:

a) we found it important to use only fresh distilled water or sterile water for injection throughout all steps in the extraction procedure involving water

b) the column pre-activation steps must be done immediately before use. The column must not be allowed to dry out before addition of the plasma solution.

c) it made no difference whether the samples were dried under an air stream or a nitrogen stream,

d) The extraction procedures of Richards et al (1) and Gutkowska et al (2) were copied and compared with our method. The main differences in these procedures versus ours lies in the solvent used to elute the ANP off the columns. Richards et al (1) used trifluoroacetic acid (TFA), while Gutkowska et al (2) used ammonium acetate. It was found that our procedure yields values that are very similar to those of Richards et al (1), while the procedure of Gutkowska et al (2) yields consistently higher values.

DOUBLE EXTRACTION PROCEDURE

We undertook several trials using this procedure in order to determine whether the ratio of "Big-ANP" (bound) to "Small-ANP" (free) could be measured within the same sample. The initial sample preparation step in the Standard Extraction Procedure, involving 4% acetic acid, is responsible for cleaving bound-ANP into free-ANP. Therefore, both bound and free ANP are measured using the Standard Extraction Procedure, but the actual ratio of bound/free ANP is unknown. We hoped to investigate not only whether ANP levels changed during pregnancy, but whether the bound/free ANP ratio changes during pregnancy.

The following procedure was developed to extract and separate the bound and free fractions.

Preparation of Samples:

-ratio of 1.0 ml plasma to 3 ml sterile water (fresh distilled or sterile water for injection)

Extraction using Sep-Pak C-18 columns (Fisher Scientific):

Column Pre-activation, steps 1-4

1) 5 ml 4% acetic acid in 85% ETOH
2) 5 ml Methanol
3) 5 ml H_2O
4) 5 ml 4% acetic acid in H_2O
5) Add plasma solution -- COLLECT run-off, Fraction #1 (this fraction should contain the bound, or "Big" ANP)
6) 2 x 3 ml H_2O wash
7) COLLECT the eluted fraction with 3 ml 4% acetic acid in 85% ETOH, Fraction #2 (this fraction should contain the free-ANP)
8) Both fractions dried under an air stream
9) Reconstitute Fraction #1 in 0.5 M acetic acid, using the same 1:3 ratio as before, and incubate for 3 hours at 4°C. This cleaves the bound-ANP into free-ANP.
10) Repeat steps 1 through 7, using new columns. Collect elutant at step 7 only. This fraction should now contain free-ANP, representative of what was originally bound-ANP.

Several trials were performed employing this method, using the same plasma pool for each trial. Unfortunately, the results obtained were ambiguous, and different with each new assay. Sometimes the free ANP fraction would be higher

than the bound fraction, and other times the opposite would occur. This should not have been the case when using the same plasma pool or each trial. It may be that this method can be made to work, however we decided to abort our efforts to make it work, and use the standard extraction procedure which we found to be more reliable.

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